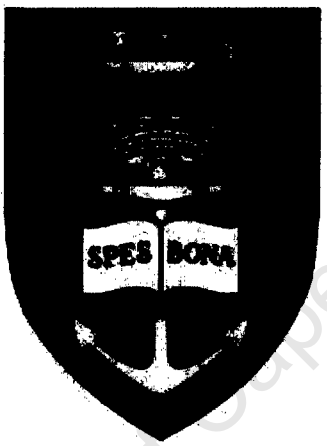
Novel chemotherapeutic agents for oesophageal cancer

Londiwe Mandisa Khuzwayo



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In the

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Supervisor: Ass. Prof. Denver Hendricks

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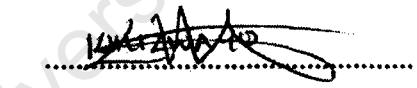
Signature	
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Table of Contents

Abbreviations

ATP	Adenosine – 5`- triphosphate
BCA	Bicinchoninic Acid
BSA	Bovine Serum albumin
°C	Celsius
CDK	Cyclin dependent kinase
CIS	Cisplatin
CXCR2	Interleukin 8 receptor
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DOX	Doxorubicin
FACS	Fluorescent activated cell sorting
FAS	TNF receptor super family member
FCS	Foetal calf serum
IGF1R	Insulin-like growth factor -1 receptor
ROS	Reactive oxygen species
γH2AX	Phosphorylated histone H2AX
OC	Oesophageal cancer
%	Percentage
SCC	Squamous cell carcinoma
mM	Millimolar
RNA	Ribonucleic acid
PARP	Poly (ADP- ribose) polymerase

NADPH	Nicotinamide adenine dinucleotide phosphate
TNFR	Tumour necrosis factor receptor
TrxR	Thioredoxin reductase
Trx	Thioredoxin
Prx	Peroxiredoxin
H_2O_2	Hydrogen peroxide
VDAC	Voltage dependent anion channel
Rb	Retinoblastoma protein
E2F	Elongation factor two
pRb	Phosphorylated-Rb
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
^{i}Pr	Isopropyl
PBS	Phosphate buffered saline
SEM	Standard error of the mean
PAGE	Polyacrylamide gel electrophoresis
OD	Optical density
P	Probability value
nm	Nanometre
kDa	Kilodaltons
IC_{50}	50 % inhibitory concentration
µM	Micromolar
g	Grams
ml	Millilitre
mg	Milligram
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)

RIPA	Radioimmunoprecipitation assay buffer
µg	Microgram
TBST	Tris-buffered saline with Tween 20
V	Voltage
NEM	N-Ethylmaleimide
DCFDA	Dichlorofluorescein Diacetate
KR	Kreb's Ringer
CI	Confidence interval
shRNA	Short hairpin ribonucleic acid
SD	Standard deviation

Abstract

Oesophageal cancer is ranked amongst the top three most common cancers affecting black South African males. Only about 20 % of patients diagnosed with this disease survive beyond two years because the cancer is typically diagnosed at a late stage and resistance to therapy commonly develops. Chemotherapeutic agents such as cisplatin, doxorubicin and 5-fluorouracil are currently used in the treatment of a variety of cancers, including oesophageal cancer. Although these agents have been part of our therapeutic repertoire for many years, there are several side effects that have been associated with their use in cancer therapy. Thus there is a need to develop novel chemotherapeutic agents with improved activity and less severe side effects. In this project, twelve compounds, consisting of 5 platinum dichloride complexes, four iminophosphine ligands and three gold (I) chloride complexes that were synthesised by a PhD student in our laboratory were tested for anticancer activity and the mode of action of these compounds was also explored. The cell viability assays revealed that the gold (I) compounds were most potent, when compared to the platinum compounds or the free ligands, with IC_{50} concentrations ranging from 5 to 30 μM. Only modest changes in cell morphology were observed in the cells treated with gold (I) compounds and a decline in cell migration was observed for compound 95 only. The decline in cell number was associated with DNA damage (assessed as an increase in γH2AX) and an arrest in the cell cycle at the S phase. Treatment with the gold (I) compounds led to the inhibition of Thioredoxin reductase activity, suggesting that ROS levels in the cells had increased. Furthermore, treatment with the gold compounds was associated with the induction of apoptosis. This suggests that the gold compounds blocked cell proliferation by processes that involved the production of ROS, DNA damage and the induction of apoptosis. Treating CXCR2 and IGF1R knockdown cells with the novel compounds did not improve efficacy against the cancer cell lines.

Chapter 1

Oesophageal cancer

1.1 Epidemiology

Presently, cancer is the second most common cause of death globally, affecting both developing and developed countries with no definitive cure.[1] In 2008 an estimated 12.4 million cases of cancer were diagnosed worldwide and 7.6 million cancer deaths were reported. More than 70 % of these deaths occured in developing countries.[2]

Oesophageal cancer (OC) is ranked amongst the top ten most common and lethal cancers in the world.[3] The incidence of OC differs geographically and with ethnicity, where high frequencies have been reported for certain parts of China, Iran, South Africa, France and Italy.[4,5] In 2002 China and South Africa had the world's highest age standardised incidence rates for this disease of 27.4 and 19.7 per 10^5 population, respectively. Furthermore global statistics indicate that males have higher susceptibility to OC than females (as seen in Figure 1).[6,7,8,9]

Oesophageal cancer is the third most common cancer affecting South African males and the most dominant cancer amongst black South African males.[10] The Transkei region (Eastern Cape) of South Africa has one of the highest incidence rates in Africa.[10,11]

A study conducted in 10 magisterial areas around the Eastern Cape and Kwa-Zulu Natal (1998 – 2002) revealed the age standardised rate for all cancers to be 72.8 per 10^5 in males and 59.1 per 10^5 in females. Oesophageal cancer accounted for 31.3 per 10^5 in males (43 % of all cancers) and 18.0 per 10^5 in females.[12]

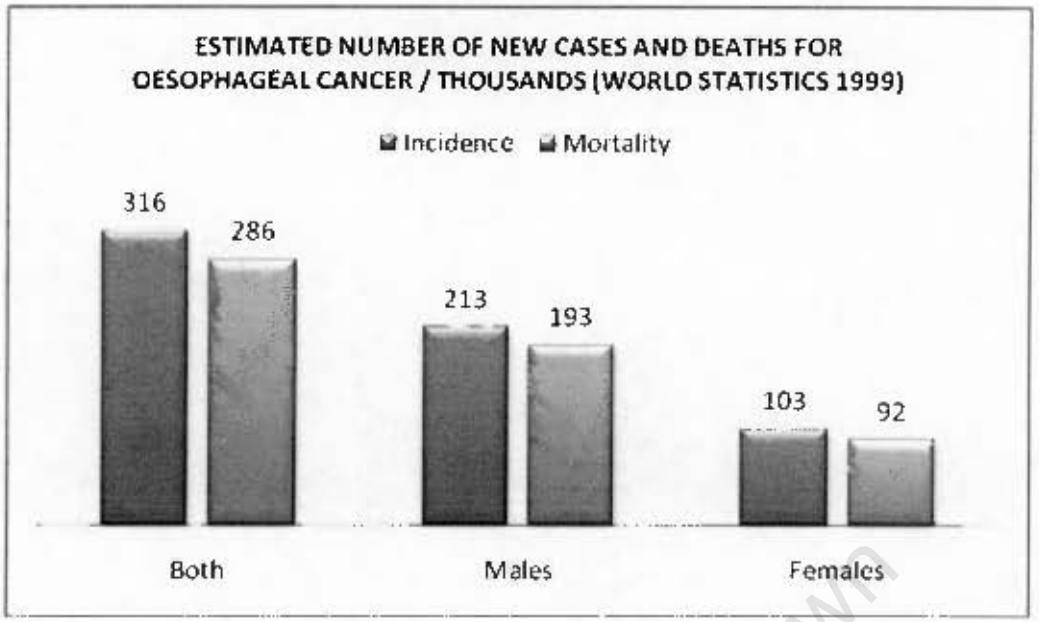


Figure 1: Comparison of incidence and mortality rates in males and females for oesophageal cancer worldwide. Males have higher susceptibility to OC than females (213 per 10^5 in males and 103 per 10^5 in females). But both sexes have extremely high mortality rates. Numerical figures were adapted from Parkin *et al.* (Global cancer statistics 1999).[11]

1.2 Histology

Oesophageal cancer exists in two histological subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC). [3,13,14] Adenocarcinoma is more common in the USA; while squamous cell carcinoma, which constitutes more than 80 % of all OC cases worldwide is most prevalent in developing countries.[15] This study focuses mainly on SCC which is the major histological subtype found amongst black South African males.

SCC develops from the middle or the lower third of the oesophagus (Figure 2A). [6,16] The tumour develops from the inner layer (epithelial cells) of the oesophagus and invades deeper into the oesophagus as it continues to grow (Figure 2B). The poor diagnosis associated with SCC leads to very few treatment options and a varying 5 year survival rate of 10 – 20 % depending on the tumour stage of development and the patient's response to therapy.[5,17,18,19]

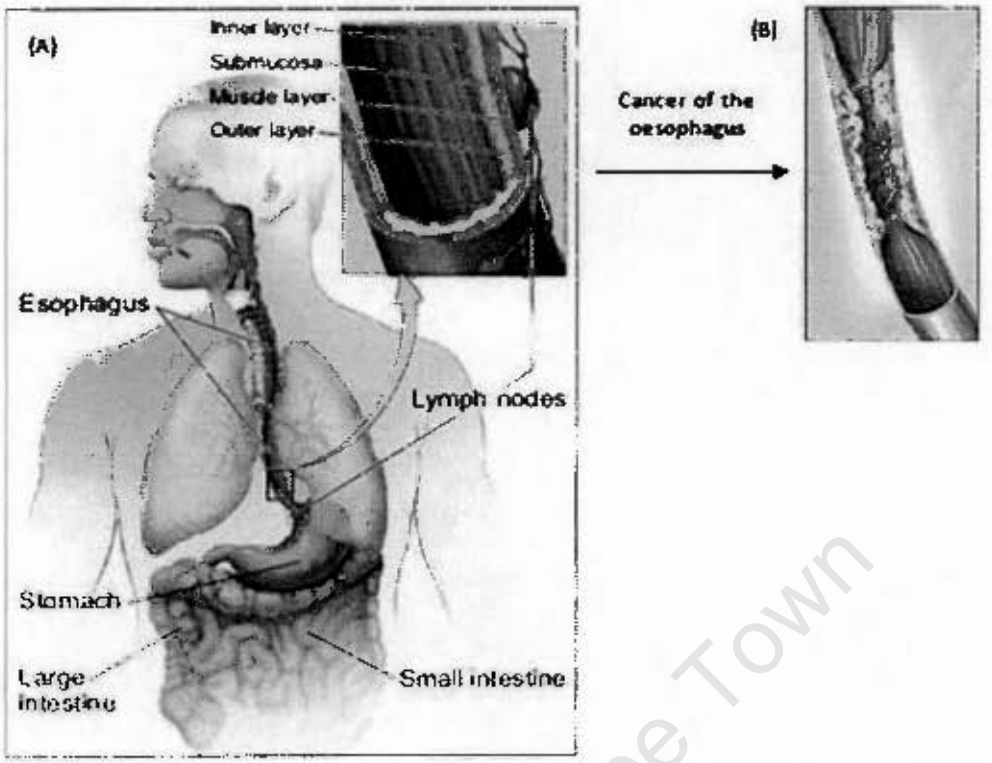


Figure 2: Section of the oesophagus prone to the development of squamous cell carcinoma (SCC). (A) The four epithelial layers of the oesophagus and surrounding organs. (B) SCC of the oesophagus.

[figure (A) adopted from (http://www.cancer.gov/cancertopics/wyntk/esophagus/page2) and figure (B) modified from (http://digestivedisease.uthscsa.edu/esophagealstent.asp)]

1.3 Therapeutic approaches

Generally, in developing countries, only 20 % of patients with oesophageal cancer would qualify for curative treatment (which includes radiotherapy, surgery and chemotherapy) while the other 80 % are suitable for palliative therapy (for example brachytherapy and stent placement) because of the late stage of diagnosis in most cases. [35,36] Palliative treatment, involves inserting a stent or tube into the oesophagus to prevent it from closing as a result of tumour growth.

Because of the aggressive nature of oesophageal cancer, it is important to develop strategies aimed at reducing morbidity and mortality associated with this cancer. This may be achieved by reducing exposure to risk factors that are associated with the development of oesophageal cancer and these include the consumption of extremely hot beverages, local dietary and cultural practices and a combination of tobacco smoking and alcohol. In addition diagnosis of OC at an early stage increases the likelihood of successful treatment and lastly by adopting better therapeutic approaches, through the development of effective chemotherapeutic agents.

As part of the drug development process, natural products from a wide range of sources (plant, algae and marine invertebrates) and chemically synthesised compounds, have been investigated for anti-tumour activity to produce more effective chemotherapeutic agents. In most cases, during cancer therapy, pharmaceuticals are used in combination with radiotherapy, surgery and other strategies aimed at palliating the effects of OC.

Due to the complications associated with surgery[22–24], combination therapy is considered more effective than monotherapy. If the tumour has metastasised, surgery alone may not be beneficial and thus adjuvant or induction therapy is required post or prior surgery.[25] In cases where the cancer has spread to the lymph nodes, radiotherapy is necessary post-surgery to minimise the risk of recurrence.[26] Chemotherapy is usually administered prior to surgery. Currently available drugs include many forms of natural derivatives and inorganic compounds. However these compounds have been associated with several side effects and this is a major drawback in cancer therapy.

1.4 Natural products

Thirteen years ago, Cragg *et al.* (1997) pointed out that more than 60 % of anticancer agents in current use then were natural product derivatives. [27–29] Natural derivatives are synthesised from secondary metabolites (usually toxic) which often function as a self defence mechanism for the organism against herbivores, pests and pathogens. However, some of these secondary metabolites may also contribute odours, tastes and colours in plants.[30] Secondary metabolites may be extracted from any part of the organism. [31,32] Many of the secondary metabolites are derived from aromatic amino acids or shikimic acids, which are precursors of alkaloids and other aromatic metabolites.[30,33]

Some examples of natural product derived cancer therapeutic agents include compounds such as podophyllotoxin (Figure 3A), Vinca alkaloids (Vincristine) (Figure 3B), taxanes (Paclitaxel) (Figure 3C), camptothecin and their derivatives. Some of these compounds are still in use to date as cancer therapeutic agents. [34,35,36]

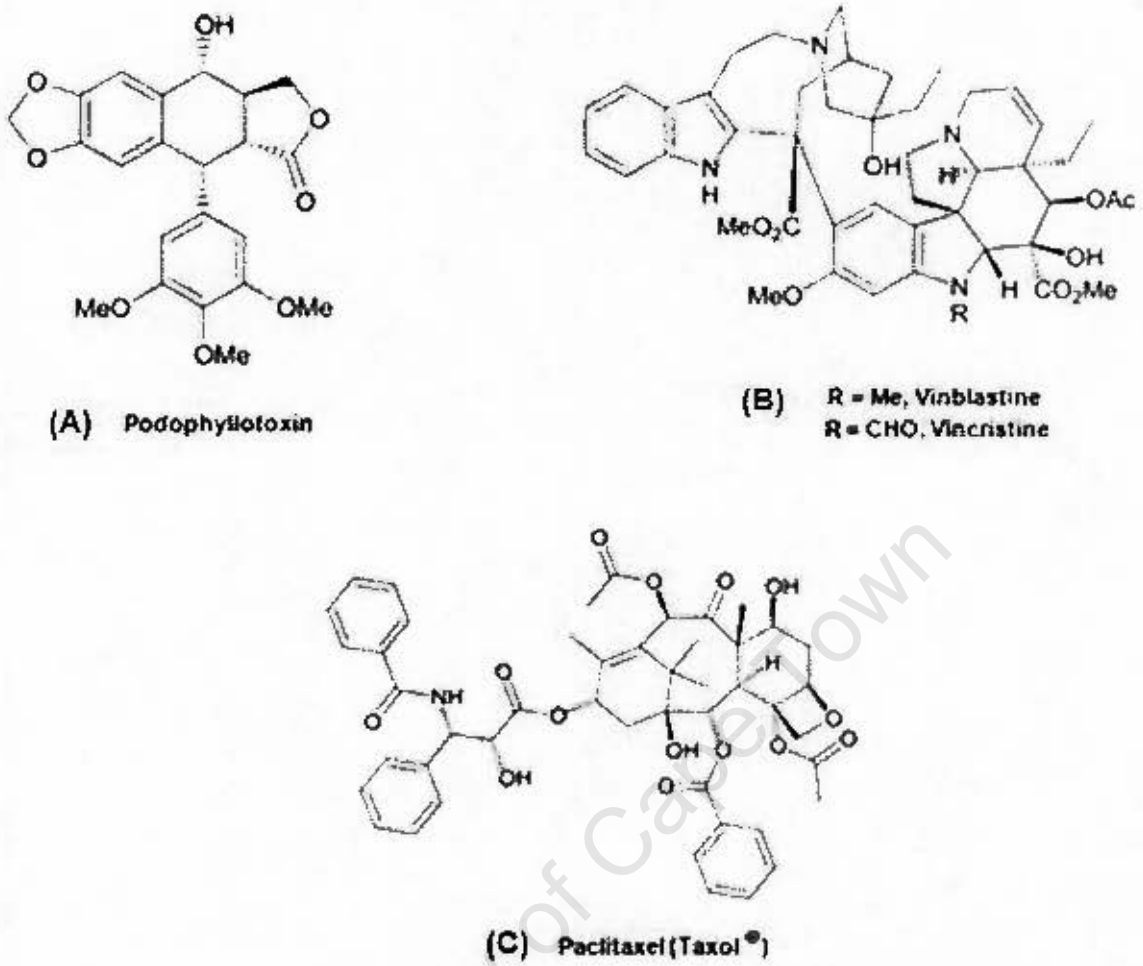


Figure 3: Structures of some chemotherapeutic agents derived from natural product sources.[35]

Although some natural products have yielded very effective anticancer drugs, there are some concerns associated with the use of natural products for drug development in medicine. These include scarcity of resources, habitat loss and degradation, as well as over-harvesting of these resources. [37] In addition to this, a study conducted by William *et al.* (2009) indicated that climate changes in the marine world may lead to local extinction of species in some regions resulting in a change in biodiversity due to species redistribution.[38] Other concerns include the ability to conserve knowledge of known medicinal properties of local natural sources while making the information more widely accessible [37]

Over the past decades there has been a decline in the production of natural pharmaceuticals in general as is evident in Figure 4 when compared to the 60 % reported by Cragg *et al.* (1997), thirteen years ago. However it is evident (as seen in Figure 4) that natural products have been a prototype in the synthesis of many novel compounds.[28,29,39,40] From 1981-2006, a total of 1184 compounds were derived from different sources in which 100 of these were anticancer agents (Figure 4). [39]

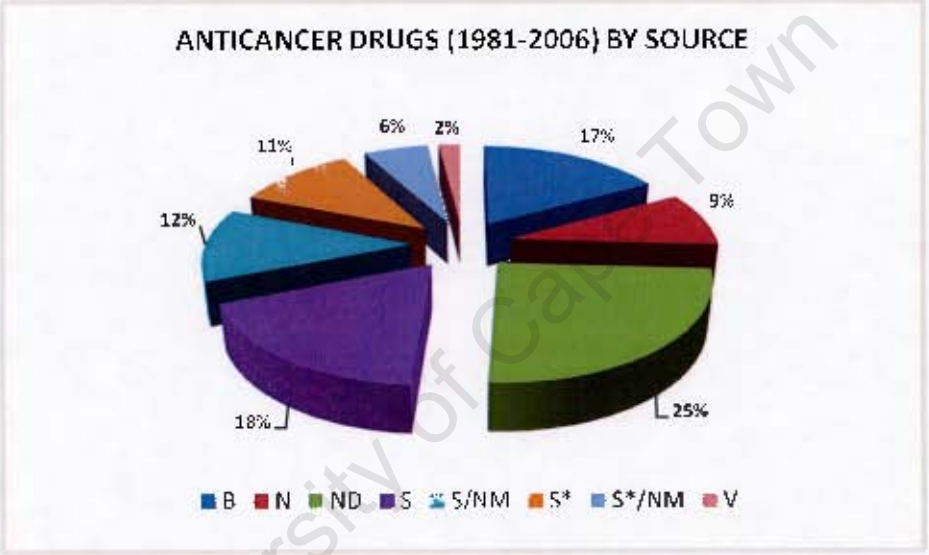


Figure 4: Anticancer compounds synthesised from 1981-2006, categorised by source. (N= 100). Codes are as in reference 50. B- biological source, N- natural product, ND- derived from natural product, S- totally synthetic from random screening, S*- totally synthetic with natural product pharmacophore, NM- Natural product mimic, V- Vaccine.

Fortunately there are a significant number of effective cancer therapeutic agents that have been developed through chemical synthetic processes that do not involve natural products. Many of these compounds are currently still in use clinically, suggesting that this route to drug development may still hold promise for development of new and effective cancer chemotherapeutic agents. Furthermore, unlike natural products which were associated with

scarcity of resources and low product yield, synthetic agents are more advantageous because production is in bulk and there is large variation of resource and product.

1.5 Inorganic compounds

The platinum complex cisplatin (*cis*-diamminedichloridoplatinum(II)) (Figure 5a) and its second and third generation analogues, carboplatin-(*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)) (Figure 5b), and oxaliplatin (*trans-R,R*-cyclohexane-(1,2-diamine)oxalatoplatinum(II)) (Figure 5c) represent a family of metal compounds that have proven to be extraordinarily useful as chemotherapeutic agents and are still in current use against several cancers globally. [41–43]

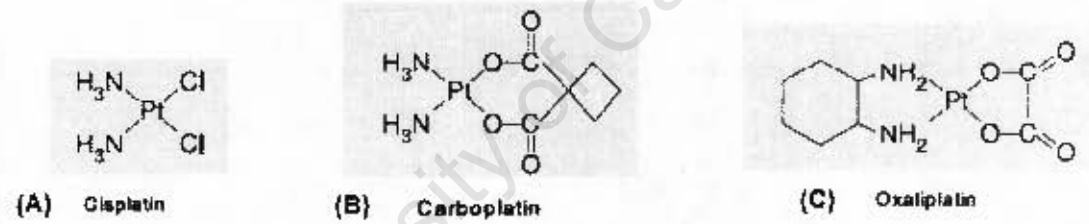


Figure 5: Structures of platinum compounds used as cancer chemotherapeutic agents.[44]

Current evidence in the literature suggests that cisplatin has been one of the most commonly used therapeutic agents in the treatment of oesophageal cancer, with its use continuing to date.[43,45–48] Cisplatin activity is dependent on the chloride concentration. In the blood the chloride concentration is about 100 mM and thus cisplatin is only slightly active. Inside the cell though, the concentration is much lower (3 mM) leading to the complete activation of cisplatin.[49] Once in a low chloride aqueous solution, the chloride separates from platinum and the active platinum reacts with nucleophilic groups present in the amino acid side chains and

purine bases of DNA and RNA.[49,50] Cisplatin interacts with DNA forming DNA adducts. It has been suggested that only 1 % of cisplatin reacts with DNA, forming DNA adducts while the rest reacts with other cellular components such as RNA, proteins, membrane phospholipids, cytoskeletal microfilaments, and thiol-containing molecules. [51–54]

DNA adducts result from different types of cross-links as illustrated in Figure 6, formed as cisplatin reacts with DNA. The 1,2 intrastrand cross-link [cis-Pt(NH_3)$_2$d(pGpG)] (Figure 6b) accounts for 65 % of the total cisplatin-DNA adducts formed while only 25 % are attributed to the 1,2 intrastrand cross-link [cis-Pt(NH_3)$_2$d(pApG)] (Figure 6c).[41,43,51,55]

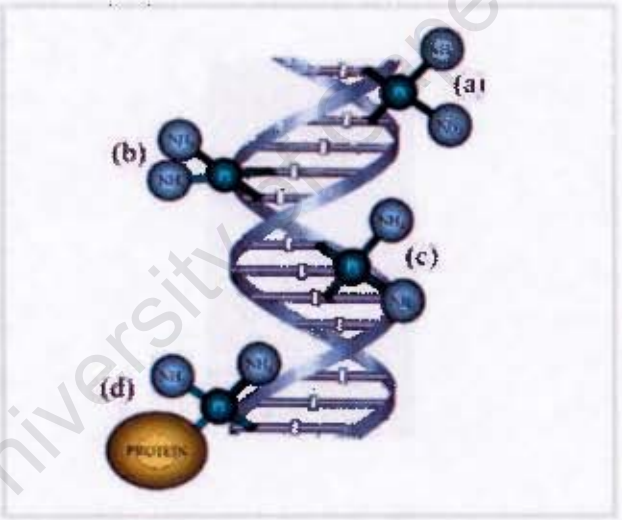


Figure 6: Cross-linking of cisplatin with DNA. (a) Intrastrand cross-link (b) 1,2 intrastrand cross-link (c) 1,2 intrastrand cross-link (d) protein- DNA cross-link.[54]

The DNA adducts are repaired by nucleotide excision repair (NER), but poor DNA repair results in the formation of DNA double strand breaks which induce cell cycle arrest. Cisplatin then exerts its cytotoxicity by inhibiting DNA synthesis and initiating apoptosis via the p53/p73 pathways, which are regulatory pathways that cells employ to monitor the integrity of genomic DNA.[41,56,57]

Despite the side effects such as nephrotoxicity, nausea and vomiting, cisplatin remains a widely used chemotherapeutic agent for solid tumours.[58] Given its success, several other platinum based anticancer complexes have been synthesised and tested in clinical trials and some (eg.carboplatin) have been shown to be more stable than cisplatin.[41,50,59] Aside from the side effects described above, one other major drawback associated with platinum-based treatment is drug resistance which has been shown to be induced via several mechanisms.[49,60]

These include decreased intracellular drug accumulation and increased induction of intracellular thiols (glutathione and metallothionein), which are synthesised when cells are subjected to stress or in response to accumulation of heavy metal ions.[49,61,62] DNA repair has also been implicated in contributing to drug resistance as a result of an up-regulation of DNA repair proteins. A study conducted by Chu *et al.* (1990) showed an increased expression of the DNA repair protein, xeroderma pigmentosum group E binding factor (XPE-BF), in cisplatin resistant cells when compared to normal cells.[63] More recently, other non-platinum based compounds have been synthesised and tested for anticancer activity and these include ruthenium, titanium, iron, cobalt and gold complexes.[64–68] These novel metal-based compounds may kill cancer cells by different pathways than cisplatin, thus increasing the repertoire of drugs at our disposal for treating patients with cancer.

1.6 Gold compounds

Gold exists in 7 different oxidation states and only gold (0), gold (I) and gold (III) are stable under aqueous and biological conditions. [69] Because gold (I) and gold (III) are readily reduced by mild reducing agents they are less stable than gold (0). Furthermore, gold (I) is thermodynamically more stable than gold (III) even though they are both soft cations with a high affinity for soft ligands.[69,70] Evidence in the literature suggests that gold compounds are

transported by binding to Cys^{34} of albumin. The process involves the conversion of gold complexes to albumin complexes [Albumin-S' + $Et_3PAuSAtg$ >> Albumin-S-Au-Pet3 + AtgSH] through a series of oxidative reactions that see the gold molecule interacting with selenoenzymes leading to its cytotoxic effects. [70,71]

Gold (I) compounds have a d^{10} closed-shell configuration, which allows linear, trigonal and tetrahedral coordination.[70]

Thiolates and phosphine complexes represent the most investigated gold (I) pharmaceuticals. Auranofin, a phosphinegold (I) thiolate complex used to treat rheumatoid arthritis, was also found to possess antitumour activity.[72–74] Auronofin induces its cytotoxic effect by inhibiting selenoenzymes such as Thioredoxin reductase. This leads to mitochondrial membrane permeability, the release of Cytochrome c and eventually apoptosis.[75]

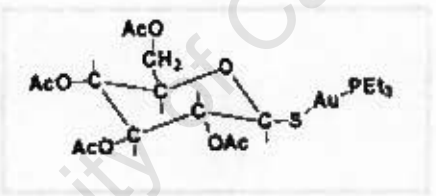


Figure 7: Structure of Auranofin.[76]

The discovery of the anticancer properties of auranofin lead to the synthesis of many other gold (I) compounds, such as [$Au(PPh_3)$(Hxspa)] and [$AuPPh_3)_2$(xspa)], which have been tested for anticancer activity and found to have cytotoxic effects on cancer cell lines.[77,78]

Gold (III) complexes display structural similarities to platinum (II) complexes (as seen in Figure 8) because they are isoelectronic (d^8 configuration) and they possess square-planar geometries and thus gold (III) complexes have also been investigated for their anticancer properties.[65]

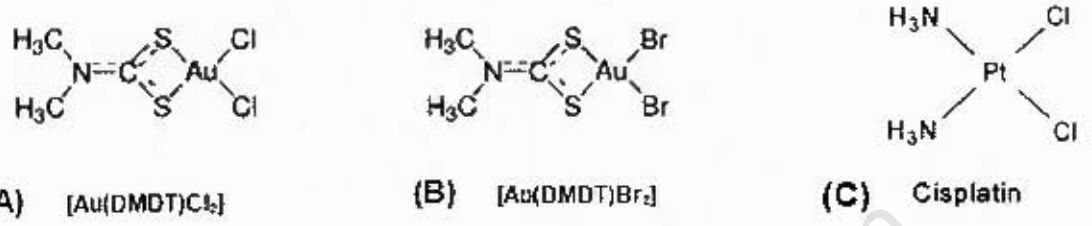


Figure 8: Structural representation of gold (III) compounds (A and B)[65] and platinum (II) compound cisplatin (C)

Gold (III)-dithiocarbamato derivatives such as [Au(DMDT)Cl_2] (Figure 8A) and [Au(DMDT)Br_2] (Figure 8B) have been found to be more active than cisplatin (Figure 8C) and were also observed to be cytotoxic to cisplatin resistant cells, suggesting that although similar to cisplatin in structure, they induce their anticancer activity via a mechanism different from that of cisplatin.[65] Dithiocarbamates are bidentate chelating ligands that are very stable once complexed to the metal ion, due to their chelating effect. The presence of a chelating dithiocarbamato is said to contribute to the reduced nephrotoxity of the compounds due to their minimal interaction with thiol containing biomolecules.[45] Regardless of the structural similarities, gold (III) dithiocarbamato complexes exert their anticancer activity through a mechanism different from that of platinum (II) compounds. Instead of targeting the DNA and forming DNA adducts, gold (III) compounds target the proteasome. Inhibition of the proteasome leads to the accumulation of ubiquitinated proteins, an up-regulation of p27 (inhibitor of the cell cycle) and an induction of apoptosis.[45,79]

This indicates that metal-based chemotherapeutic agents exert their effect by activating a variety of biological pathways ultimately leading to cell death. An understanding of the mechanism of action of these compounds may facilitate the development of more effective chemotherapeutic agents.

In a previous study conducted in our laboratory, a fellow student (Dr. Harry Chiririwa) had synthesised a series of metal-based compounds that were further characterised in this project to better understand how these novel compounds induced cancer cell death. In the next section of this review, we considered some of the biological pathways frequently affected by chemotherapeutic agents, to provide a background for some of the studies that were conducted in this project.

Tumour cells are characterised by deregulated cell proliferation and suppressed cell death and both these processes involve the activation of several complex pathways.[80–82] Therapeutic agents induce cell death in cancer cells via apoptosis, necrosis or autophagy (as seen in Figure 9). Generally in the literature, there has been a tendency to develop cancer therapeutic agents that induce cell death via apoptosis and not via necrosis (which is associated with the induction of inflammation) or autophagy (which has been implicated in promoting survival of cancer cells under stressful conditions).[83–85] The mechanisms that lead to the activation of cell death involve a wide range of processes including the generation of reactive oxygen species (ROS), cell cycle arrest, mitochondrial dysfunction or the alteration of pro-apoptotic and anti-apoptotic proteins. Therefore, when characterising novel therapeutic agents, it is important to identify and understand the pathways by which these agents induce cell death and inhibit cell proliferation.

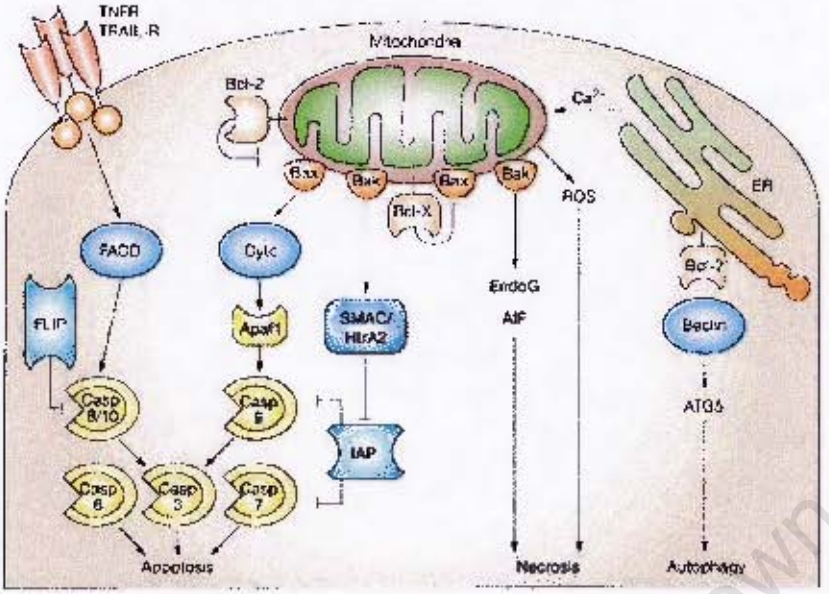


Figure 9: Diagrammatic representation of some of the pathways that lead to cell death in eukaryotes. Three modes of cell death are represented. Apoptosis may be initiated through the activation of death receptors (TNFR/TRAIL-R) or via the mitochondria, leading to the release of Cytochrome c (Cytc) and the activation of caspases (Casp) which cleave Poly (ADP-ribose) Polymerase (PARP). Similarly the activation of death receptors leads to the activation of several other processes such the recruitment of the adaptor molecule FADD, leading to the activation of caspases. The mitochondria are also responsible for inducing necrosis via ROS production or the activation of Apoptosis inducing factor (AIF). An autophagic response may be initiated in the endoplasmic reticulum (ER) and this leads to the activation of downstream effectors such as Beclin.
Figure obtained from, http://www.medscape.com/viewarticle/540893_2

1.7 Autophagy

The ubiquitin-proteasome and autophagy systems are essential for maintaining balance between biosynthetic and degradative processes for normal cell growth and development.[84] Autophagy is essentially a survival mechanism. When cells undergo nutrient and growth factor depletion, cells exit the cell cycle and autodigest long-lived proteins and damaged organelles. This leads to the recycling of lipids, carbohydrates and amino acids, to produce macromolecules or to facilitate oxidation in the mitochondria to maintain cellular ATP.[85,86] Therefore if autophagy is used as a strategy by cancer cells to escape the cytotoxic effects of

therapeutic drugs, blocking this pathway with specific drugs may provide an attractive approach to kill cancer cells.[85] Decreased expression of Beclin-1, a marker for autophagy, has been associated with the development of a tumourigenic phenotype.[85] Further more, an increase in Beclin-1 expression has been shown to inhibit cell proliferation and decrease tumourigenic potential.[86,87] However, the information regarding the role of autophagy in tumourigenesis and therapy is controversial [88–91] and thus experimental evidence requires careful analysis.

1.8 Apoptosis

Apoptosis or "programmed cell death" is an essential process in development and in maintaining homeostasis in self-renewing tissues of normal cells. Defects in this process could prolong the life span of cells, a condition associated with neoplasia.[92,93] Apoptosis may be initiated via the activation of death receptors such as Tumour necrosis factor receptor (TNFR) or TNF receptor super family member (FAS), mitochondrial dysfunction or via ROS generation.[92,94] The initiation of apoptosis via death receptors involves the activation of adaptor proteins such as FADD in the presence of stimuli (therapeutic agents or irradiation). This then leads to the activation of caspases 8/10, which further cleaves other caspases and target proteins to induce apoptosis (as seen in Figure 9).[94] As previously mentioned, apoptosis may be induced via the mitochondria as a result of a build up of ROS or through the alteration of pro and anti-apoptotic proteins (Figure 9). This is associated with the release of Cytochrome c which activates Apaf1 and Caspase 9, leading to the initiation of apoptosis.[95]

The process of apoptosis is divided into early and late stage apoptosis.[96,97] A study conducted by Prete *et al.* (2002) suggested that mRNA degradation is an early stage apoptotic event that

is common to all apoptotic pathways.[98] The process of apoptosis is adenosine triphosphate (ATP) dependent and evidence in the literature suggests that ATP depletion and loss of membrane integrity occur during late stage apoptosis.[83,97,99–103] As part of the process of apoptosis, a nuclear protein poly (ADP-ribose) polymerase (PARP) is typically proteolytically cleaved, generating a 89 kDa fragment from a 116 kDa precursor form. Both the precursor and the cleaved products are detectable by Western blot analysis.[104–106]

1.9 The mitochondria

In addition to generating ATP, the mitochondria play a crucial role in maintaining the cellular redox state, calcium levels and facilitating apoptosis.[93,107–110] Deregulation of any of these processes will lead to a change in mitochondrial membrane potential ($\Delta\psi$). Furthermore, deregulation of pathways that are responsible for maintaining the cellular redox state may lead to mitochondrial damage and elevated ROS levels.[107] Gold compounds have been shown to alter the mitochondrial membrane potential ($\Delta\psi$) by inhibiting thiols (such as Thioredoxin reductase) that are essential for regulating ROS levels (Figure 11).

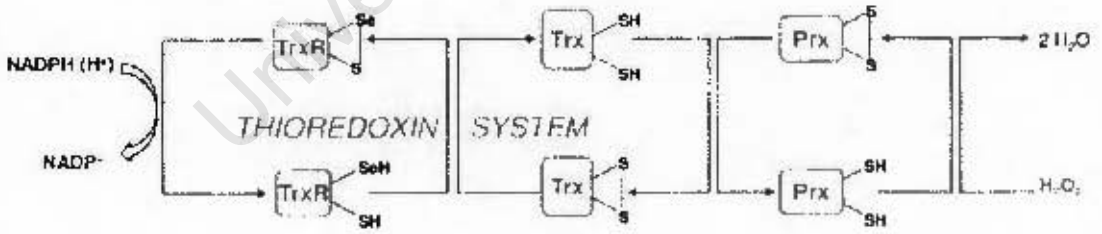


Figure 11: The Thioredoxin reductase system. Reduced NADPH initiates the reduction of Thioredoxin reductase (TrxR – SeH) which leads to the reduction of Thioredoxin (Trx) and Peroxiredoxin (Prx). Prx is a ROS scavenger. The reduced form of the proteins are indicated by SeH/ SH and the oxidised by Se/S.[111]

From Figure 11, it is observed that in the presence of NADPH, Thioredoxin reductase (TrxR) facilitates the reduction of Thioredoxin (Trx), which in turn reduces Peroxiredoxin (Prx) which inhibits the build-up of hydrogen peroxide (H_2O_2). It has been proposed that gold compounds alter the Thioredoxin system by inhibiting the activity of Thioredoxin reductase, resulting in the rapid accumulation of oxidised Prx. The accumulation of oxidised Prx leads to an accumulation of H_2O_2 species, which increase mitochondrial permeability and this leads to the release of Cytochrome c and the activation of Caspases, ultimately resulting in apoptosis.[111]

Other factors that lead to changes in mitochondrial membrane potential include the alteration of pro- and anti-apoptotic proteins, mediated by the Bcl-2 family of proteins. A study conducted by Shimizu *et al.* (1999) indicated that, in the presence of death stimuli, the pro-apoptotic protein Bax translocates to the mitochondrial membrane. There, it interacts with the voltage dependent anion channel (VDAC), leading to a loss of mitochondrial membrane potential ($\Delta\psi$) and the release of Cytochrome c. Furthermore, the study showed that the presence of Bcl-X_L, an anti-apoptotic protein inhibited the interaction between VDAC and Bax and thus inhibiting the induction of apoptosis.[112,113] In addition to mitochondrial permeability, the cell cycle also plays a major role in inducing apoptosis, as indicated above with cisplatin.

1.10 The cell cycle

The cell cycle (Figure 12) is an important regulatory process in eukaryotes that is required for accurate duplication and transmission of DNA information from the parent cell to daughter cells.[114] The cell cycle consists of DNA check points (G1 and G2) that monitor DNA integrity during cell division. The Rb/E2F transcription complex is located at the restriction point of the cell cycle. DNA replication occurs at the S phase and cell division, which results in the formation of two identical daughter cells, occurs at the M/Mitosis phase. In the absence of growth factors or mitogens, cells remain dormant or in the quiescent state (G0) and once the necessary chemical stimuli (growth factors and mitogens) are present, the cells exit G0 and enter into G1. The retinoblastoma (Rb) protein is then phosphorylated by cyclin D/cdks, leading to the dissociation of the Rb/E2F complex and progression of the cell cycle.[114,115]

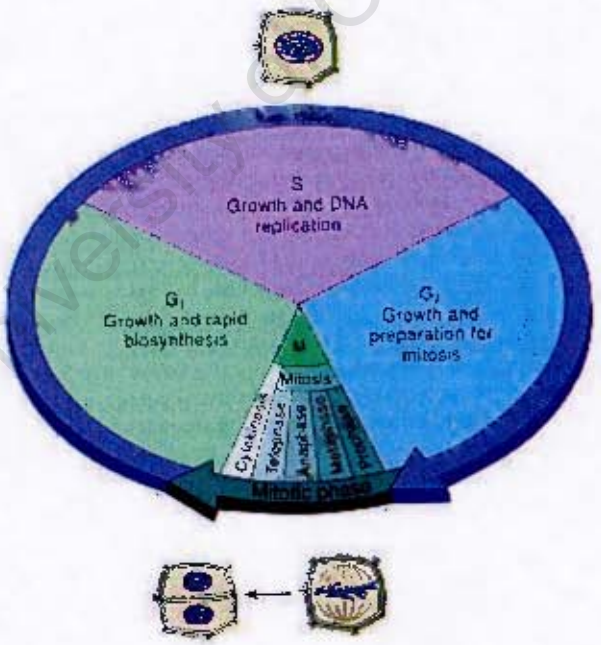


Figure 12: Schematic representation of the stages of the cell cycle. This figure was adopted from (http://www.centergrove.k12.in.us/cms/lib4/IN01000850/Centricity/Domain/500/Cell%20Cycle.jpg)

The G1 or gap1 checkpoint, checks the cells for the presence of DNA damage or mutations before entering the S phase for DNA replication.[81] The G2 checkpoint ensures that all the DNA was duplicated during the S phase and that the chromosomes are ready for separation into two daughter cells. The cell then enters into M phase. Cyclin dependent kinases (CDKs) are activated periodically and they are responsible for mediating cell cycle progression.[115]

In cancer cells, cyclin D expression is frequently up-regulated and thus there are elevated levels of pRb, resulting in continuous cell division. Furthermore the G1 and G2 check points are disrupted, giving way to uncontrollable cell proliferation and the transmission of damaged or mutated DNA to daughter cells.[114,116] Chemotherapeutic agents often act by inducing DNA damage, thus inhibiting cell cycle progression. This cell cycle arrest is followed by DNA repair, however, if the DNA can not be repaired, apoptosis is induced. In many cases cell cycle inhibition has been associated with high levels of ROS which react with the DNA causing DNA damage.[107]

In this review, I have briefly reviewed some important biological pathways that are frequently activated by chemotherapeutic agents, eventually leading to cell death. I have also shown that the late presentation of oesophageal cancer is associated with a poor prognosis, with most patients having a life expectancy of less than two years at the time of diagnosis.[117]

Thus there is an enormous amount of work that still needs to be done in improving cancer therapy. Therefore there is continuous need to develop novel, more effective and less toxic therapeutic agents. We propose that the synthesis and characterisation of novel metal-based complexes may hold some promise for the development of novel chemotherapeutic agents.

1.11 Aim

The aim of this study was to screen several novel compounds for cancer cell growth inhibitory properties, to characterise their mechanism of action and to test their effect in a panel of cancer cell lines and normal fibroblast cells.

1.12 Objectives

1. Were to determine the cancer cell growth inhibitory activity of several metal complexes and their ligands against a panel of cancer cell lines, compared to normal fibroblast cultures. These compounds were synthesised by a PhD student (Dr Harry Chiririwa) at our laboratory.

2. To characterise the mechanism of action of the active compounds. This objective was achieved by:

    - Testing for apoptosis
    - Testing for ROS generation
    - Monitoring DNA damage

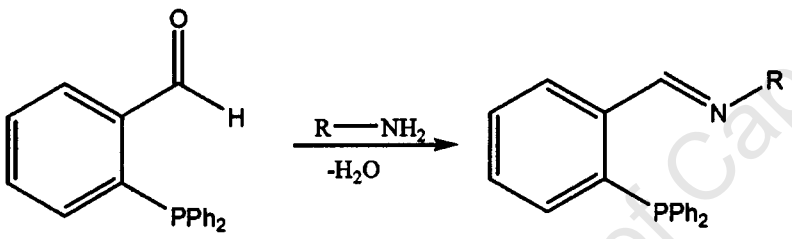
3. To determine the effects of the compounds on the cell lines where the CXCR2 and IGF1R pathways had been altered (WHCO1 and WHCO6 knockdown cells). The knockdown cell lines were prepared by Dr Luke Esau.

Chapter 2

Materials and methods

2.1 Reagents and compounds

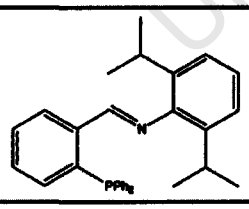
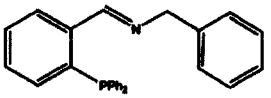
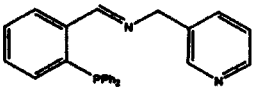
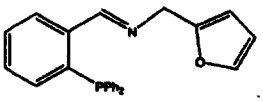
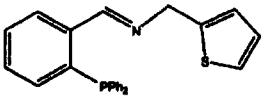
For this project five iminophosphine ligands were synthesised (as shown in Figure 2.1) by a PhD student (Dr Chiririwa) at our laboratory, using *o*-diphenylphosphinobenzaldehyde as a precursor. The iminophosphine ligands were obtained at a yield of ≥75 % and their structural representations are shown on Table 2.1.



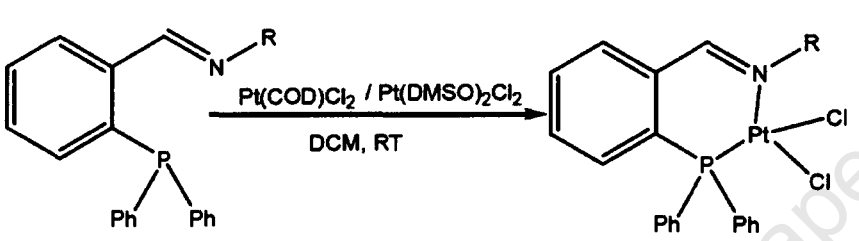
R = 2,6 ^{i}Pr C_6H_3	60
R = $(CH_2)C_6H_5$	61
R = $(CH_2)C_5H_4N$	62
R = $(CH_2)C_4H_3O$	63
R = $(CH_2)C_4H_3S$	64

Figure 2.1: The synthesis of novel iminophosphine ligands[118]

Table 2.1: Structures of the ligands.[118]

60 	61 	62 
63 	64 	

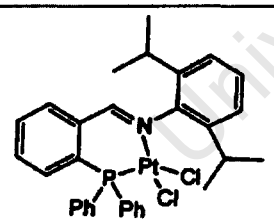
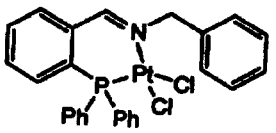
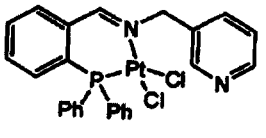
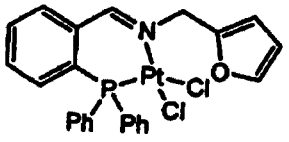
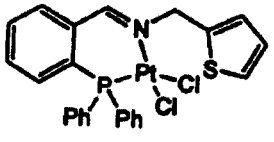
Iminophosphine ligands consist of 'hard' nitrogen and 'soft' phosphine donor atoms, a property that enables the ligands to bind soft metals ions such as Pt^{+2}, Au^{+} and Ag^{+}.[118,119] For this project five platinum dichloride complexes were synthesised by reacting ligands 60 to 64 with either Dichloro-(1,5-cyclooctadiene)-platinum(II) [$Pt(COD)Cl_2$] or Platinum(II) dimethylsulfoxide complex [$Pt(DMSO)_2Cl_2$] as seen in Figure 2.2. Structures for the resultant complexes are shown in Table 2.3. The highest yield of 72 % was obtained for compound 84. Compounds 85 to 88 gave yields of 68 %, 70 %, 71 % and 70 % respectively.[118]



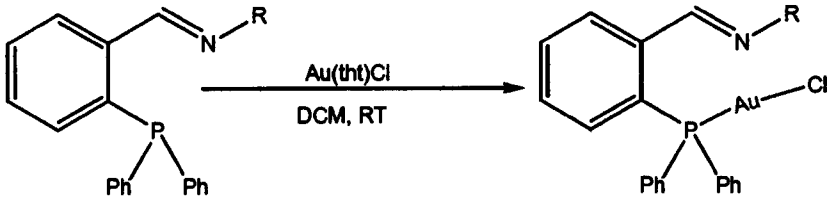
R = $(CH_3)_4(CH)_2C_6H_4$	84
R = $(CH_2)C_6H_5$	85
R = $(CH_2)C_5H_4N$	86
R = $(CH_2)C_4H_3O$	87
R = $(CH_2)C_4H_3S$	88

Figure 2.2: The synthesis of novel platinum dichloride complexes[118]

Table 2.2: Structures of the platinum dichloride complexes[118]

84 	85 	86 
87 	88 	

In addition to the synthesis of platinum complexes, two gold (I) chloride complexes were synthesised from reacting ligands 63 and 64 with Chlorotetrahydrothiophen gold (l) Au(tht)Cl (Figure 2.3). Complexing the other ligands (60, 61 and 62) with the gold metal ion resulted in the formation of insoluble gold complexes which could not be characterised further.



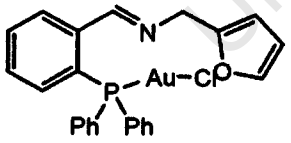
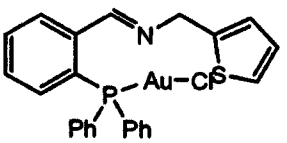
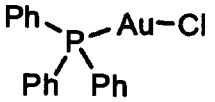
R = $(CH_2)C_4H_3O$ 93

R = $(CH_2)C_4H_3S$ 94

Figure 2.3: The synthesis of novel gold (I) chloride complexes[118]

Linear coordination for compounds 93 and 94 was confirmed by X-ray crystallography.[118] An additional gold (I) complex (95) was synthesised by reacting Au(tht)Cl with triphenylphosphine, however this compound is not novel, like the other two compounds (93 and 94). The structural representations for all three gold complexes are shown on Table 2.4.

Table 2.3: Structures of gold (I) chloride complexes [118]

93	94	95
		

The components of the buffers used in the following section are found in appendix B.

2.2 Cell culture

The oesophageal squamous cell carcinoma cell lines (WHCO1, WHCO5, WHCO6, KYSE70, KYSE150, KYSE410 and KYSE450), cervical cancer cell lines (CaSki, HeLa, SiHa and ME180) as well as two breast cancer cell lines (MCF-7 and MDA-MB-231) and two normal fibroblast cell cultures (FG_0 and DMB), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100 U/ml Penicillin, 100 µg/ml Streptomycin and 10 % (v/v) heat inactivated Foetal Calf Serum (FCS) (GIBCO, Paisley, USA). WHCO1, WHCO5 and WHCO6 cells were obtained as a generous gift from Dr. Veale[120] and the KYSE70, KYSE150, KYSE410 and KYSE450 cell lines were obtained from Dr. Yutaka Shimada (University of Toyama, Toyama, Japan) and purchased from the DSMZ cell bank. The normal skin fibroblasts, FG_0 and DMB cells were obtained as a generous gift from Dr. A. D. Marais (Groote Schuur Hospital, Cape Town) and the other cell lines were purchased from the American Type Culture Collection (ATCC) (Rockvile, MD, USA). Cell cultures were maintained at 37°C in a humidified atmosphere of 5 % CO_2 and culture dishes were kept at 70 % - 95 % confluency. The cells were sub-cultured with 0.05 % (v/v) Trypsin-EDTA solution and neutralised with DMEM (1:1 ratio). For long term storage, cells were re-suspending in freezing medium [10 % (v/v) DMSO (# R36/38, MERK, Darmstadt, Germany), 20 % (v/v) FCS, and 70 % (v/v) DMEM] and 1ml aliquots were frozen at -80°C before being transferred to liquid nitrogen.

2.2.1 Mycoplasma test

To test for mycoplasma contamination, all cell lines were subjected to a mycoplasma test regularly. Cells were cultured in Penicillin and Streptomycin (P/S) free DMEM for one week, after which they were plated onto coverslips and incubated at 37°C overnight. Cells were fixed in fixative (1:3 glacial acetic acid: methanol) and stained with 0.5 µg/ml Hoeschst fluorescent DNA-binding stain (# 33258, Sigma, USA). The coverslips were mounted and visualised on the Axiophot fluorescence microscope (Zeiss, Germany).

2.3 MTT assays

2.3.1 IC_{50} determination

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a Tetrazolium salt which is used to measure the metabolic rate of cells through the reduction of the MTT to formazan in the mitochondria of metabolically active cells. MTT assays were conducted on a series of oesophageal, cervical and breast cancer cells lines as well as on normal fibroblast cells. The experiments were conducted in 96 well plates in triplicate, for 8 different concentrations (see Appendix A for treatment tables). Cancer cells ($3x10^3$) and normal cells ($5x10^3$) were plated per well in 90 µl DMEM and incubated at 37°C overnight. 10X drug concentrations in medium containing 0.2 % (v/v) DMSO (# D2650, Sigma, USA) were prepared in a concentration range as shown on the treatment tables (Appendix A). 10 µl of the 10X drug dilutions was added to the cells which were then incubated at 37°C for 48 hours. Cells were observed under the Telaval 31 microscope (Zeiss, Germany) before they were incubated for 4 hours at 37°C with 10 µl of 5 mg/ml sterile MTT reagent (# M2128, Sigma, USA). Subsequently, 100 µl of solubilisation reagent was added to the cells and incubated at 37°C for 16 hours, to dissolve the formazan crystals. The absorbance were

determined at 595 nm on the BioTek EL800 microplate reader (USA) using the Gen5 software, readings were exported as an Excel file and the data was analysed using Graphpad prism.

2.3.2 Cell growth assay

WHCO1 oesophageal cancer cells ($1.5x10^3$) were plated per well in 90 µl DMEM and incubated at 37°C overnight in 96 well plates. 10X drug concentrations were prepared using 0.5X, 1X and 2X the IC_{50} concentration. After each time point (Time 0, 24, 48, 72, 96, 120 hours after treatment) 10 µl of MTT reagent was added to the cells and incubated for 4 hours at 37°C. 100 µl of solubilisation reagent was added to the cells and incubated at 37°C for 16 hours. The absorbance was determined at 595 nm on the BioTek EL800 microplate reader using Gen5 software. Readings were exported as a Microsoft Excel file and the data was analysed using Microsoft Excel.

2.3.3 Treating IGF1R and CXCR2 knockdown cells

MTT assays were conducted on WHCO1 and WHCO6 cell lines, either untransfected (wild-type) or transfected with shRNA targeting Insulin-like growth factor receptor I (IGF1R) or CXCR2, or a control shRNA sequence (Ctrl). These stably transfected cell lines were generated by a PhD student in our laboratory (Dr Luke Esau). Prior to the experiments conducted in this project, the expression status of CXCR2 and IGF1R was determined by Western blot analysis and the results shown in chapter 5, for the WHCO6 cell line were obtained by Dr Luke Esau in our laboratory. The experimental procedures were carried out as described above (2.3.1).

2.4 Cell morphology assay

WHCO1 cells ($1x10^5$) were plated in 60 mm plates and incubated at 37°C overnight. Cells were treated with 1X or 2X the IC_{50} concentration, for 12, 24 and 48 hours. After each time point pictures were taken on the Telaval 31 phase contrast microscope using the Mitocam 2500, 5.0MP Live Resolution camera (Motic®, China).

2.5 Cell migration assay

WHCO1 cells ($1x10^6$) were plated per well in 6 well plates and incubated overnight at 37°C. A vertical scratch across the cell layer was made using a p200 pipette tip, after which the medium was changed to remove dead cells and detritus. 10 µg/ml of Mitomycin C (# 4287, Sigma, USA), an inhibitor of cell proliferation, along with drug (IC_{50} concentration) was added to the cells. The scratch width was measured at 0, 8 hours and 24 hours after treatment. For each condition 3 different views were captured using the phase contrast settings on the Axiovert 200M fluorescence microscope (Zeiss, Germany) and 3 different measurements were taken per view.

2.6 Cell cycle profile

This assay was conducted in triplicate in 60 mm plates. WHCO1 cells ($3x10^5$) were plated and incubated overnight at 37°C. Cells were treated with 1X and 2X the IC_{50} concentration for 12, 24 and 48 hours. Cells were harvested at the indicated time points by trypsinisation and centrifuged at 200X g for 5 minutes. The pellet was resuspended in 2 ml of media and cells were counted with a Telaval 31 microscope using a haemacytometer. Cells were fixed in 8 ml of ice-cold 100 % (v/v) ethanol (EtOH) and stored at -20°C for a minimum of 24 hour to a maximum of 2 weeks. The cells were pelleted by centrifugation at 200X g for 5 minutes. The pellet was washed in 1 ml 1xPBS and samples were centrifuged at 200X g for 5 minutes. The pellet was resuspended in 1 ml 1xPBS and an amount equivalent to the overall lowest cell count was aliquoted into an eppendorf vial for each sample. Samples were centrifuged at 200X g for 5 minutes and the supernatant was discarded. For every $1x10^6$ cells, 200 µl of PBS containing 50 µg/ml of RNAse A (# 84524822, Boehringer Mannheim, Germany) was added. Samples were incubated at 37°C for 15 minutes. For every $1x10^6$ cells, 1 ml of PI (# P4170, Sigma, USA) staining solution was added to the PBS + RNAse solution, 20 minutes before FACS analysis. Samples were analysed on a Facs calibur (Germany) using the cell Quest Pro. programme and data was exported and analysed using ModFit software.

2.7 Thioredoxin reductase activity

Previous studies have shown that gold compounds inhibit Thioredoxin reductase activity in various cell types (See discussion and Figure in Chapter 1, Figure 11).[111,121,122] To establish if the novel gold (I) compounds had the same mode of action, experiments were conducted to determine the effects of these compounds on the Thioredoxin Reductase system in oesophageal cancer cells. Auranofin, which has been shown to inhibit the activity of Thioredoxin Reductase in both the mitochondria and the cytoplasm, was used as a positive control. [75]

2.7.1 Extracting total Thioredoxin reductase (TrxR) protein

This procedure was carried out essentially as described by Cox *et al.* (2008), with a few modifications. WHCO1 cells ($1x10^6$) were plated in 100 mm plates and incubated overnight at 37°C. Cells were treated with 5 µM of compounds 93 and 95, 6 µM of compound 94 and 1 µM of auranofin (# El-206-0/00, Enzo life sciences), for 30 minutes. Cells were then detached in trypsin, which was neutralised with medium. Cells were harvested and centrifuged at 200X g for 5 minutes. Samples were washed with lysis buffer and pelleted. The pellet was resuspended in 200 µl of lysis buffer suplemented with 1 % (w/v) CHAPS (# 220201, CalBioChem, Canada) and 1X complete protease inhibitor (# 11697498001, Roche, Germany). Samples were then centrifuged at 10000X g at, 4°C, for 10 minutes. The supernatant was transferred into a clean eppendorf vial.

2.7.2 Fractionation assay

This assay was conducted as described in Cox *et al.* (2008) with a few modifications. Because Thioredoxin Reductase is found both in the mitochondria and cytoplasm, a fractionation assay was conducted to separate mitochondrial and cytoplasmic extracts. WHCO1 cells ($12x10^6$) were plated on 145 mm plates and incubated overnight at 37°C. The cells were treated with auranofin, compound 93, compound 94 and compound 95 as above, for 30 minutes. Cells were detached in trypsin, which was neutralised with DMEM. Cells were then harvested and centrifuged at 200X g for 5 minutes. The pellet was resuspended in 1xPBS and centrifuged at 600X g, 4°C for 5 minutes. The supernatant was removed and the pellet was frozen at -80°C for 10 minutes, to weaken the cell membrane. The pellet was then thawed and resuspended in mitochondrial isolation buffer supplemented with 150 µg/ml of digitonin (# D141, Sigma, USA) and 1X complete protease inhibitor. Samples were incubated on ice for 10 minutes and then sonicated for 1 minute to ensure complete cell lysis. Samples were centrifuged at 1000X g, 4°C for 10 minutes to remove whole cells, nuclei and debris. The supernatant was transferred into a clean pre-chilled eppendorf and centrifuged at 12000X g for 20 minutes. The supernatant (cytosolic fraction) was transferred into a clean eppendorf and the pellet (mitochondrial fraction) was resuspended in 200 µl of mitochondrial isolation buffer containing 2 % (w/v) CHAPS and 1X Protease inhibitor.

2.7.3 Thioredoxin reductase activity assay

In the presence of Thioredoxin Reductase (TrxR), 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) is reduced to 5-thio-2-nitrobenzoic acid (TNB); in an NADPH dependent reaction.[111] Using this principle, endogenous Thioredoxin Reductase activity was determined. The NADPH (# N6505, Sigma, USA) was dissolved in Phosphate EDTA (PE) buffer and the DTNB (# D8130, Sigma, USA) was dissolved in DMSO. The experiment was conducted in

duplicate. Protein sample (60 µg) from either the total TrxR extraction or fractionation assay was mixed with 180 µl of 0.24 mM NADPH and 6 µl of 5 mM DTNB. The change in absorbance (at 5, 10, 20 and 60 minutes after the first reading) was measured at 405 nm on the BioTek EL800 microplate reader using Gen5 software, and data was analysed using Microsoft excel.

2.8 Protein quantification

The Bicinchoninic Acid (BCA) assay is a commonly used assay in protein quantitation. In principle, the presence of protein results in the reduction of Cu^{+2} to Cu^{+}, which further reacts with the BCA to give off an intense purple colour that can be measured at 562 – 595 nm wavelength. [123,124] The colour intensity is dependent on the amount of protein that is present and the incubation time. The BCA assay kit (# 23225, Pierce, Rockford, USA) was used to quantify the protein samples and a 2 mg/ml bovine serum albumin (BSA) stock was used to generate a dilution series of BSA standards (2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.2 mg/ml and 0.1 mg/ml), which were used to determine the protein concentration for unknown samples. The absorbance was measured at 595 nm on the BioTek EL800 microplate reader using Gen5 software.

2.9 Western blot analysis

2.9.1 Antibodies

Primary antibodies were purchased from Santa Cruz Biotechnology, Cell signaling (USA) and Sigma and all primary antibodies and incubation conditions that were used are shown in Table 2.4. The donkey anti-goat secondary antibody (# SC2020, Santa Cruz Biotechnology, USA), goat anti- rabbit (# 170-6515, BioRad, USA) and goat anti-mouse (# 170-6516, BioRad) HRP-conjugated secondary antibodies were used at dilutions of 1:4000 in 2.5 % (v/v) milk, 1:5000 in 5 % (v/v) milk and 1:1000 in TBS-Tween, respectively.

Table 2.4: Primary antibody conditions used for Western blot analysis. Interleukin 8 receptor beta (CXCR2β), Insulin-like growth factor -1 receptor beta (IGF1Rβ), Poly ADP-ribose polymerase 1/2 (PARP), Peroxiredoxin (PRX), phospho-histone 2AX (γH2AX).

Antibody (Ab)	Catalog no.	Company	Dilution	Diluent	Type
β-tubulin (h-235)	SC9140	Santa Cruz Biotechnology	1:1000	TBS-Tween	Polyclonal
CXCR2β (s-16)	SC22661	Santa Cruz Biotechnology	1:2000	TBS-Tween	Polyclonal
IGF1Rβ (c-20)	SC713	Santa Cruz Biotechnology	1:1000	5 % Milk	Polyclonal
PARP (h-250)	SC7150	Santa Cruz Biotechnology	1:1000	5 % Milk	Polyclonal
PRX1	SAB2101877	Sigma	1:1000	2 % Milk	Polyclonal
PRX2	SAB2101878	Sigma	1:1000	2 % Milk	Polyclonal
PRX3	AV52341	Sigma	1:2000	2 % Milk	Polyclonal
γH2AX (s-139)	2577S	Cell signaling	1:2000	TBS - Tween	Monoclonal

2.9.2 Protein extraction

2.9.2.1 Protein extraction for PARP and γH2AX detection

WHCO1 cells ($1x10^5$) were plated in 60 mm plates and incubated at 37°C overnight. Cells were treated with compounds 93, 94 and 95 at 1X or 2X the IC_{50} concentration, for 12, 24 and 48 hours. Doxorubicin (5 μM) was used as a positive control. Proteins were extracted in 60 μl radioimmunoprecipitation assay (RIPA) buffer (recipe is in the appendix) supplemented with 1X complete protease inhibitor cocktail and 1 mM Na_3VO_4 phosphatase inhibitor (# S6008, Sigma, USA). Cells were scraped off the dish using a cell scraper and the lysate was sonicated for 10 seconds. The cell lysate was centrifuged at 11000X g for 12 minutes at 4°C to remove cell debris and the supernatant was transferred into a clean eppendorf vial. The protein concentration was determined using the BCA kit and protein samples were stored at -80°C.

2.9.2.2 Protein extraction for IGF1R and CXCR2 detection

WHCO1 cells, WHCO1 IGF1R shRNA knockdown cells, WHCO1 Ctrl shRNA cells, and WHCO1 CXCR2 shRNA knockdown cells were cultured in 100 mm plates to about 80 % confluency. Proteins were extracted in 200 μl of RIPA supplemented with 1X complete protease inhibitor cocktail and 1 mM Na_3VO_4 phosphatase inhibitor. Protein samples were then processed as described above (2.8.2.1).

2.9.3 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein sample (5 µg) was added to 5X Laemmli buffer for the detection of PARP and yH2AX, while 20 µg of protein was used for the detection of IGF1R and CXCR2. Samples were denatured at 95°C for 5 minutes prior to loading. Protein samples were electrophoresed through a 4 % stacking gel and 10 % resolving gel. The voltage was set at 180 V and samples were electrophoresed for 1 hour.

2.9.4 Immunoblotting

Proteins were transferred onto a Hybond™-ECL™ nitrocellulose membrane (Amersham Life sciences, UK) at 4°C for 1 hour at 100 V. The transfer tank was kept on ice to maintain a cool environment.

Membranes were blocked in 5 % (w/v) fat free powder milk (dissolved in TBS-Tween) for 1 hour at room temperature on a shaker. The primary antibody was added and incubated overnight at 4°C on a shaker.

Membranes were subjected to three 10 minute washes with TBS-Tween at room temperature on a shaker to remove unbound antibody. The secondary antibody was added and incubated for 1 hour at room temperature on a shaker. The 10 minute wash steps were repeated before detection.

2.9.5 Immunodetection

Super signal detection reagent (# 34080, Pierce, USA) was used to detect protein and in instances where the protein signal was too low, LumiGlo Reserve (# 54-71-00, KPL Inc, USA), which is a much more sensitive detection reagent was used, according to the manufacturers instructions. X-ray film was exposed to membrane with chemiluminescent

substrate for varying exposure times. The X-ray film was developed until bands were clearly visible before being fixed.

2.9.6 Stripping the nitrocellulose membrane

Because most of the primary antibodies used were produced in the same animal model, membranes were stripped using beta-mercaptoethanol stripping buffer. Unlike glycine stripping buffer which removes the secondary antibody only, beta-mercaptoethanol stripping buffer removes both primary and secondary antibodies.[125] Membranes were incubated with beta-mercaptoethanol stripping buffer (pre-heated to approximately 40°C) for 15 minutes at room temperature. Membranes were rinsed briefly in TBS-Tween and blocked with 5 % (w/v) fat free milk for 30 minutes at room temperature on a shaker. Membranes were then processed as previously described.

2.9.7 Peroxiredoxin (Prx) Western blotting

Prx is a ROS scavenger and a downstream target of Thioredoxin Reductase. Prx exists in 6 different forms of which Prx1 and 2 are specific to the cytoplasm and Prx3 is specific to the mitochondria. [126,127] To determine the downstream effects of TrxR inhibition, Prx1, 2 and 3 protein levels were detected using non-reducing Western blot conditions, which are suitable for detecting both the oxidised and reduced forms of the Prx proteins. WHCO1 cells ($2x10^5$) were plated in 60 mm plates and incubated overnight at 37°C. Cells were treated with 1 μM, 3 μM and 6 μM auranofin in DMEM for 30 minutes, 2 and 4 hours. DMEM was removed from the cells and 100 μl of NEM (# 04259, Sigma, USA) buffer supplemented with 10 μg/ml Catalase (# C1345, Sigma, USA) and 1X Protease inhibitor was added to the cells. Plates were incubated at room temperature for 15 minutes. 100 μl of 2 % (w/v) CHAPS was added to the plate containing NEM buffer and cells were scraped off the plates using a cell

scraper. To ensure efficient cell lysis, samples were further homogenised by pippeting up and down several times. Protein samples were quantified as described above and 20 µg of protein was combined with beta-mercaptoethanol-free loading dye and electrophoresed on a 12 % SDS-PAGE gel. Proteins were transferred to a nitrocellulose membrane and blots were processed as described previously.

2.10 ROS assay

Elevated levels of ROS are indicative of cellular stress that may potentially lead to the inhibition of the cell cycle or induction of apoptosis (amongst other responses).[128] The Thioredoxin reductase system is one of the major pathways involved in regulating ROS and effectively inhibiting the Thioredoxin reductase pathway will lead to an increase in ROS levels. To determine the effects of compounds 93, 94 and 95 on ROS levels, a ROS assay was established, where Doxorubicin (# D1515, Sigma, USA) was used as a positive control. [128] WHCO1 cells ($1x10^5$) were plated per well in 6 well plates and incubated overnight at 37°C. Medium was removed from the plates and the cells were washed with warm Kreb's Ringer (KR) buffer. Cells were incubated with 50 µg/ml DCFDA (# D6883, Sigma, USA) at 37°C for 30 minutes. Cells were treated for 30 minutes with 1X or 2X the IC_{50} concentration, the drug was added directly to the DCFDA and KR buffer solution. Cells were harvested by trypsinisation in 1 ml of trypsin which was neutralised with 200 µl of FCS. Wells were washed with 1 ml KR buffer and pooled with trypsinised cells. Cell extracts were centrifuged at 200X g for 5 minutes and the pellet was resuspended in 500 µl of KR buffer. 100 µl of sample was aliquoted into white (opaque) 96 well plates in triplicate. Fluorescence was read on a Cary Eclipse fluorescence plate reader at an excitation wavelength of 484 nm and

emission of 530 nm. The remaining 200 µl was used for protein quantitation. Data was normalised using the protein concentration.

Because reproducibility was a problem with the above protocol, a second protocol was developed.

WHCO1 cells ($2x10^4$) were plated in tissue culture white 96 well plates. The cells were incubated overnight at 37°C. Medium was removed from the cells and cells were washed in warm KR buffer. Cells were incubated with 50 µg/ml DCFDA for 30 minutes at 37°C. Cells were then treated with 1X or 2X the IC_{50} concentration for 30 minutes. Wells were washed twice with KR buffer and 100 µl of KR buffer was added to each well and plates were read on a Cary Eclipse fluorescence plate reader at an excitation wavelength of 484 nm and emission wavelength of 530 nm using the Advanced reads programme. Data was processed on Graphpad prism.

2.11 ROS Scavenger assay

To try and evaluate the effect of ROS levels on cell survival upon treatment with compounds 93, 94 and 95, a ROS assay was conducted was conducted in the absence and presence of selected ROS scavengers. The crystal violet stain was used in this assay. The amount of dye that is taken up by the cells can be measured using a 96 well plate reader, upon addition of the solubilisation solution.

WHCO1 cells ($5x10^3$) were plated per well in 96 well plates and incubated overnight at 37°C. The experiment was done in triplicate. Medium was removed from the wells and the cells were treated with increasing concentrations (0.5, 3 and 5 mM) of NAC in 90 µl DMEM for 1 hour. 10 µl of 10X drug stock was added per well and incubated for 24 hours at 37°C.

Adherent cell were fixed in 100 µl methanol per well for 10 minutes and stained with 40 µl of crystal violet stain for 20 minutes. Cells were washed by immersing plates repeatedly in a basin containing tap water to reduce background. 100 µl of solubilisation solution (50 % (v/v) acetic acid) was added to each well. After contents had been allowed to dissolve completely, the plates were read at 595 nm on the BioTek EL800 microplate reader using Gen5 software. Readings were exported as an Excel file and the data was analysed using Graphpad prism.

2.12 Statistical analysis

To evaluate the difference between treated and control samples, the t-test, one-way ANOVA (Dunnett) test as well as the Two-way ANOVA (Bonferroni) posttest, were conducted on Graphpad prism. The level of significance was set at $p< 0.05$.

Results Section

Chapter 3

Screening novel compounds for cancer cell growth inhibitory properties

3.1 Introduction

In total 13 compounds consisting of 5 ligands, 5 platinum compounds and 3 gold (I) compounds were synthesised for this project, however the experiments to follow, were conducted on twelve compounds. Only re-crystalised and pure compounds were used in the assays described below.

Cisplatin, 5-fluorouracil and doxorubicin, are chemotherapeutic agents that are currently used in the treatment of a variety of cancers including oesophageal cancer.[129,130] Because of the several side effects associated with the use of these drugs, there is an urgent need to develop novel chemotherapeutic agents, that are less toxic to normal cells, more specific to tumour cells and that induce their cytotoxic effects via mechanisms that are different from those of known chemotherapeutic agents.

One of the objectives for this project was to determine the cytotoxic activity of the novel compounds previously synthesised in our laboratory (as described in Chapter 2.11) against a wide panel of oesophageal cancer cell lines, as well as some cervical and breast cancer cell lines, compared to normal fibroblasts. The effects of these compounds on cell function were determined using the MTT assay which is a standard assay used in the drug screening process during drug discovery, to assess cell proliferation and cytotoxicity.[46,131–134] Other techniques such as flow cytometry for cell cycle analysis, as well as macroscopic observation of cell morphology by phase contrast microscopy and an evaluation of cell migration, using a scratch / wound assay, were also used during the drug screening process. There are several

studies that have highlighted the importance of developing novel chemotherapeutic agents targeted at inhibiting uncontrolled cell proliferation, angiogenesis, invasion, as well as drugs with an ability to promote apoptosis and reverse loss of cell differentiation.[80,135,136] Thus by conducting cell cycle analysis, cell morphology and cell migration assays we were able to determine the effects of the novel compounds on uncontrolled cell replication, cell differentiation and invasion, respectively, all of which are important markers of tumourigenicity.

3.2 Effects of compounds on cell viability

The indicated cells were treated with different concentrations of the compounds as indicated in Section 2.3.1 for 48 hours, and cell viability was determined. The results for the MTT assays (some typical results shown in Figure 3.1) were used to calculate IC_{50} values for the various compounds in the cells tested. Compounds with IC_{50} values above 100 μM were considered inactive, and these IC_{50} values as well as those where the IC_{50} could not be determined are reported as undefined (UD) in the tables. Although compounds with an IC_{50} value less than 100 μM were considered active, this did not mean that their level of activity and selectivity were the same. For instance, compounds with IC_{50} values of 10 μM or less were considered to be more active than those with IC_{50} values of 15 μM and above. Suggesting that if an IC_{50} value of 2 μM was obtained for cell line (a) after treatment with compound (x) and an IC_{50} value of 10 μM was obtained in cell line (b), for the same compound. This meant that compound (x) was more active in cell line (a) when compared to (b) and thus more selective in cell line (b), suggesting that 10 μM of compound (x) would be toxic to cell line (a). Thus the results were interpreted with these considerations.

The platinum compounds were first tested on a panel of oesophageal cancer cell lines (Table 3.1) and of the five platinum compounds tested, compound 84 showed the most activity against cancer cells, with an IC_{50} value of 2 μM for the WHCO1 cell line and 4 μM for the KYSE450 cell line. The rest of the compounds (85 to 88) showed little or no activity against the oesophageal cancer cell lines.

The activity of these compounds was also tested on other cell lines which included two cervical cancer cell lines and two normal fibroblast cell cultures (Table 3.2). Compounds 85 to 88 showed similar activity to that observed in the oesophageal cancer cell lines. Compound 84 showed slight selectivity in the FG_0 cells.

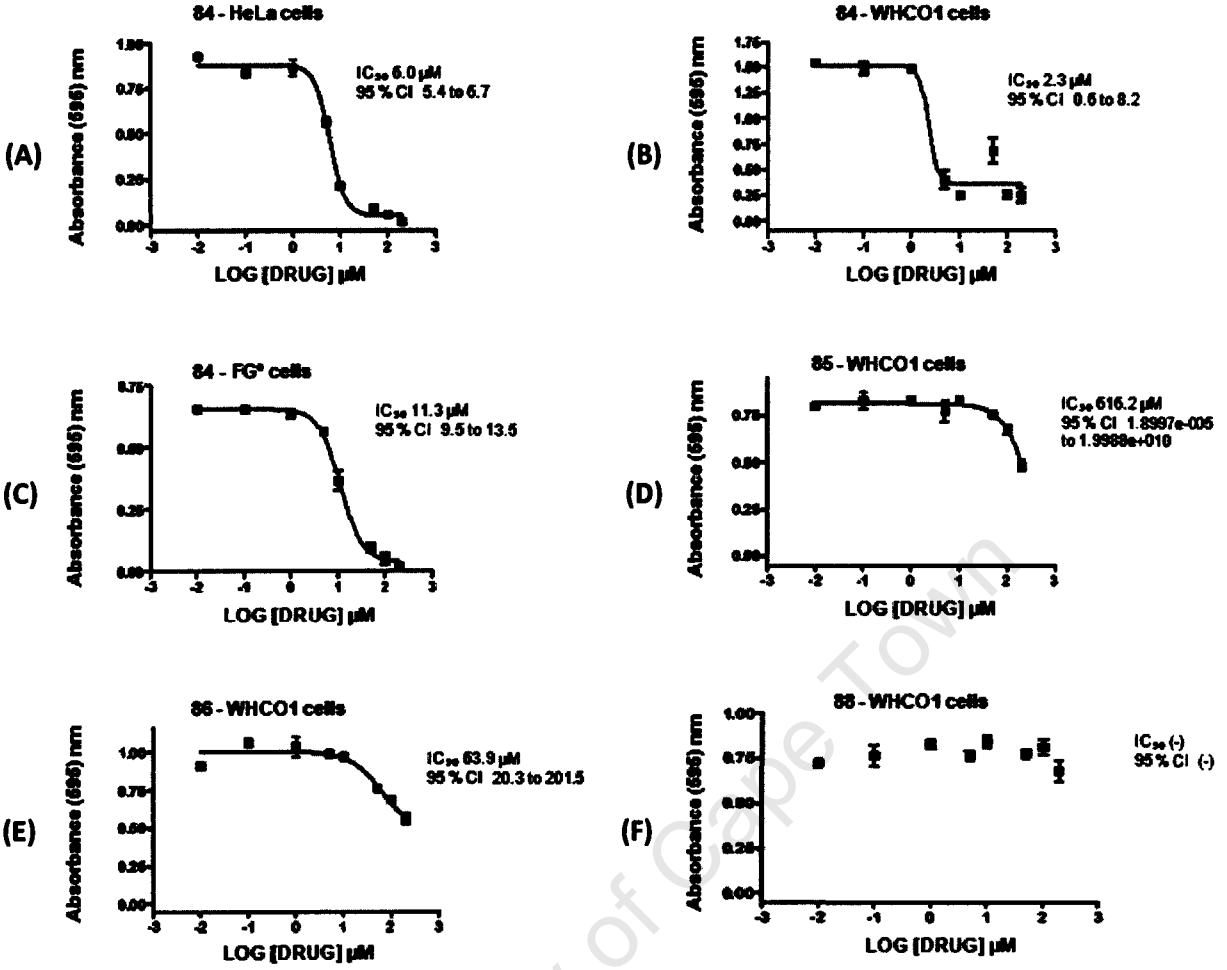


Figure 3.1: MTT results obtained after treating cells with platinum compounds. The above MTT results are a general representation of the observations made after treating various cell lines with platinum compounds. The data points represent the mean of the triplicate and the error bars represent the SEM. Cells were plated in 96 well plates and cultured for 24 hours. Thereafter 10 µl of medium containing various concentrations of compounds were added to the wells and incubated for 48 hours. The final DMSO concentration in all the wells was 0.2 %. After 48 hours, MTT reagent was added and incubated for 4 hour, followed by solubilisation reagent. After 16 hours, the absorbance of the plates was determined at 595 nm and the IC_{50} values were calculated as described in materials and methods. IC_{50} values that were less than 100 µM as represented by A, B, C and E were considered active, and those that were above 100 µM (D) or in instances where a graph could not be obtained (F) were considered inactive and are presented as undefined (UD) on the results tables.

Table 3.1

Testing the effects of platinum compounds on cell viability

Five platinum compounds were tested for their effects on cell viability by conducting MTT assays on five oesophageal cancer cell lines. Drug 84 was not tested on the cell lines indicated by a dash (-). IC_{50} values that were above 100 µM or in instances where a graph could not be obtained the results are expressed as undefined (UD). This result represents a single experiment done in triplicate and data analysis was done on graphpad.

IC_{50} µM (95 % CI)					
PLATINUM COMPOUNDS	**OESOPHAGEAL CANCER CELL LINES**				
	WHC01	**WHC05**	**KYSE150**	**KYSE410**	**KYSE450**
84	2 (0.6 to 8.2)	-	-	-	4 (3.27 to 4.53)
85	UD	UD	UD	UD	UD
86	64 (20 to 201)	UD	100	UD	UD
87	UD	70 (52 to 92)	58 (46 to 100)	100 (73 to 136)	UD
88	UD	UD	UD	83 (55 to 126)	UD

Table 3.2

Testing the effects of platinum compounds on cell viability

Five platinum compounds were tested for their effects on cell viability by conducting MTT assays on cervical cancer cell lines and normal fibroblast cultures. Drug 84 was not tested on the cell line indicated by a dash (-). IC_{50} values that were above 100 µM or in instances where a graph could not be obtained the results are expressed as undefined (UD). This result represents a single experiment done in triplicate and data analysis was done on graphpad.

IC_{50} µM (95 % CI)				
PLATINUM COMPOUNDS	**CERVICAL CANCER CELL LINES**		**NORMAL FIBROBLAST**	
	CASKI	**HELA**	**DMB**	**FG_0**
84	5 (-102 to 1.0 e^{1056})	6 (5.4 to 6.7)	-	11 (9.5 to 13.5)
85	UD	31 (13 to 75)	UD	UD
86	UD	UD	UD	UD
87	UD	57 (45 to 73)	UD	87 (53 to 140)
88	UD	UD	UD	66 (53 to 82)

In addition to platinum derivatives, non-platinum based compounds such as ruthenium, titanium, iron, cobalt and gold complexes have also been shown to possess anticancer activity.[66–68,137,138] The synthesis of non-platinum based compounds could be advantageous

for cancer therapy because other metal-based compounds may show reduced side-effects, and yet still display significant anticancer activity. Consequently, the PhD student in our laboratory had ligated all the ligands to Chlorotetrahydrothiophen [Au(tht)Cl] as shown in the materials and methods (section 2.11), but only compounds 93 and 94 were soluble (representing ligands 63 and 64) and could be tested further. In addition, compound 95 was synthesised by reacting Au(tht)Cl with triphenylphosphine. All three compounds were then tested for anticancer activity. The general overview of the MTT results obtained after treating cells with gold (I) compounds are presented in Figure 3.2.

The presence of a gold metal atom instead of a platinum metal generally appeared to enhance the activity of the compounds. IC_{50} values of 12 to 16 μM were obtained in the KYSE410 and KYSE450 cell lines (Table 3.3) which showed little or no sensitivity to the platinum compounds (Table 3.1). The gold (I) compounds showed the most activity in the WHCO1 and KYSE150 cell lines and the least activity in the KYSE70 cell line (Table 3.3). To determine if the gold compounds were active in other cancer types, MTT assays were conducted on cervical and breast cancer cell lines as well as on normal fibroblast cell cultures (Table 3.4).

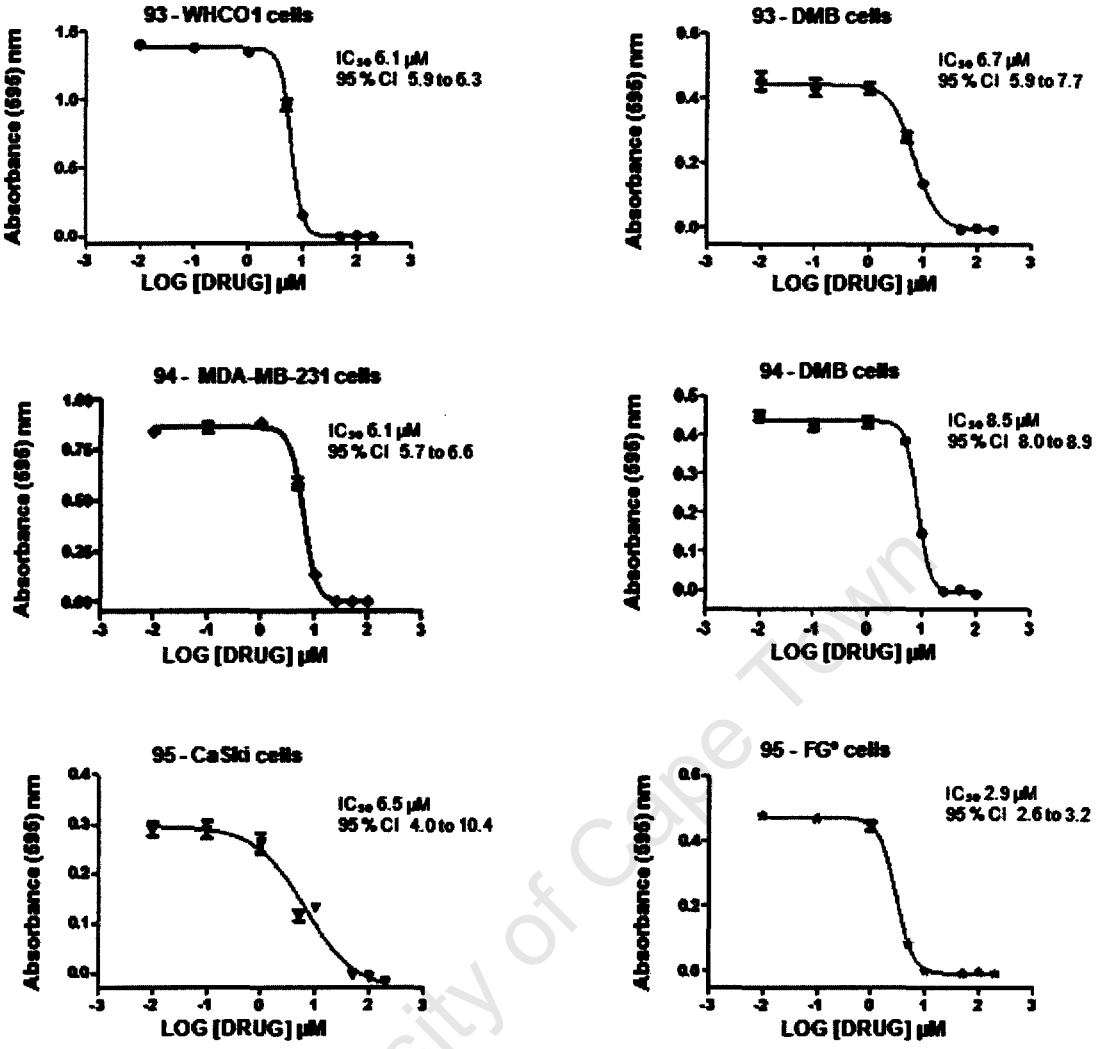


Figure 3.2: MTT results obtained after treating cells with gold (I) compounds. The data points represent the mean of the triplicate and the error bars represent the SEM. Cells were plated in 96 well plates and cultured for 24 hours. Thereafter 10 µl of medium containing various concentrations of compounds were added to the wells and incubated for 48 hours. The final DMSO concentration in all the wells was 0.2 %. After 48 hours, MTT reagent was added and incubated for 4 hour, followed by solubilisation reagent. After 16 hours, the absorbance of the plates was determined at 595 nm and the IC_{50} values were calculated as described in materials and methods. The gold compounds were observed to be generally active across all cell lines tested.

Table 3.3

Testing the effects of gold (I) compounds on cell viability

Three gold compounds were tested for their effects on cell viability by conducting MTT assays on oesophageal cancer cell lines. The 95 % confidence interval (CI) could not be obtained in the samples designated by a dash (-).This result represents a single experiment done in triplicate and data analysis was done on graphpad.

	IC_{50} µM (95 % CI)					
GOLD COMPOUNDS	OESOPHAGEAL CANCER CELL LINES					
	WHC01	WHC05	KYSE70	KYSE150	KYSE410	KYSE450
93	3 (2.5 to 3)	5 (4 to 6)	36 ( )	4 (4 to 5)	9 (8 to 11)	8 (6 to 9)
94	5 (4 to 5)	7 (6 to 8)	29 (19 to 40)	5 (4 to 5)	11 (9 to 13)	12 (10 to 15)
95	3.5 (1 to 10)	13 (5 to 30)	27 (23 to 30)	2.5 (2 to 3)	10.5 (8.5 to 13)	16 (11.5 to 22)

Table 3.4

Testing the effects of gold (I) compounds on cell viability

Three gold compounds were tested for their effects on cell viability by conducting MTT assays on three cervical cancer cell lines, two breast cancer and two normal fibroblast cultures. This result represents a single experiment done in triplicate and data analysis was done on graphpad.

	IC_{50} µM (95 % CI)						
GOLD COMPOUNDS	CERVICAL CANCER CELL LINES			BREAST CANCER CELL LINES		NORMAL FIBROBLAST	
	CASKI	HELA	SIHA	MCF-7	MDA-MB-231	DMB	FG_0
93	7 (6 to 7)	6 (5.5 to 7)	7 (7 to 8)	8 (8 to 9)	6.5 (6 to 7)	6 (5 to 7)	4 (3 to 4)
94	8 (7 to 9)	9 (8 to 10)	7 (5.5 to 10)	9 (8 to 10)	6 (5.7 to 6.5)	6 (4.5 to 7)	3 (2 to 3)
95	6.5 (4 to 10)	18 (12 to 29)	11 (10 to 12)	10 (10 to 11)	16 (11 to 24)	13 (10 to 18)	3 (2 to 3)

The gold (I) chloride compounds were effective against all the cancer cell lines tested but were similarly active in killing normal cells (Table 3.4). The CaSki cell line which showed the least sensitivity to treatment with platinum compounds was one of the most responsive cell lines to the treatment with gold (I) compounds. The non-selectivity of the gold (I) compounds against the cancer cells compared to normal fibroblasts is disappointing, but consistent with the response of WHCO1, FG_0 and DMB cells to treatment with known chemotherapeutic agents (Table 3.5). Although the novel compounds show no selectivity, it may be beneficial to further explore these findings on animal models.

Table 3.5

Testing the effects of known chemotherapeutic drugs on cell viability

Three known chemotherapeutic agents were tested for their effects on cell viability by conducting MTT assays on the oesophageal cancer cell line, WHCO1 and two normal fibroblasts FG_0 and DMB cultures. The 95 % confidence interval (CI) could not be obtained in the samples designated by a dash (-). To show reproducibility the results obtained from the first experiment and the repeat experiments are presented on this table. Data analysis was done on graphpad.

		IC_{50} µM (95 % CI)		
COMPOUNDS	**EXPERIMENT**	**WHCO1**	**FG_0**	**DMB**
DOXORUBICIN	1st experiment	0.7 (0.65 to 0.76)	UD	0.0003 (-)
	2nd experiment	0.6 (0.56 to 0.65)	Not tested	1.5 e^{-07} (-)
CISPLATIN	1st experiment	27 (22 to 33.5)	53 (36 to 79)	31 (17 to 57)
	2nd experiment	23 (18 to 29)	Not tested	53 (47 to 60)
5-FLUOROURACIL	1st experiment	30 (5 to 170)	9.8 (3 to 32)	UD
	2nd experiment	10 (3 to 32)	9 (4 to 21)	UD

Although highly active in killing cancer cells, doxorubicin appeared to be more effective in killing the DMB cells, but showed little activity against the FG_0 cells. Similar to the novel gold (I) compounds, 5-fluorouracil showed no selectivity in killing normal and cancer cells.

In order to evaluate how the presence of the metal atom contributes to drug activity, MTT assays were conducted on the uncomplexed ligands (compounds 60 – 64). The ligand for compound 85 was excluded because it was insoluble in the medium. The general overview of the MTT results obtained after treating cells with ligands is presented on Figure 3.3. Ligands with IC_{50} values similar to the ones observed in A and C were considered inactive because the IC_{50} values did not correspond to the observations made on the graph and in same cases the 95 % confidence interval (CI) could not be obtained (Figure 3.3). However ligands with IC_{50} values similar to the ones shown in B and D were considered active.

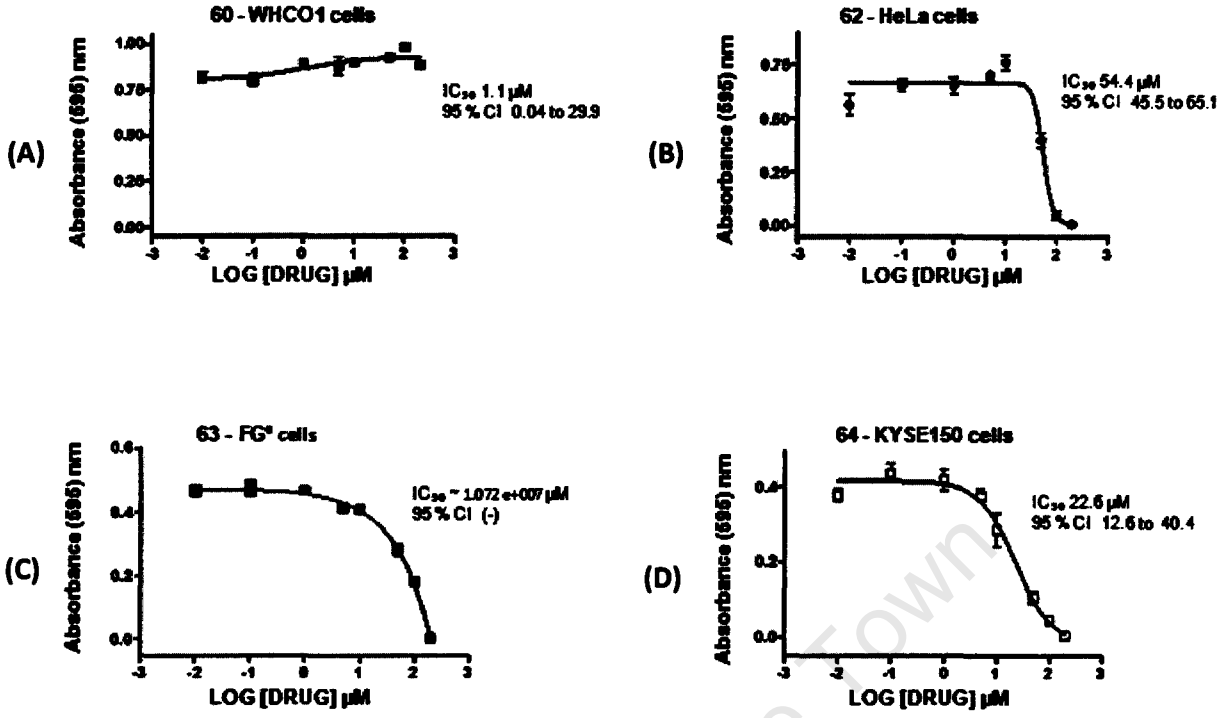


Figure 3.3: MTT results obtained after treating cells with ligands. The data points represent the mean of the triplicate and the error bars represent the SEM. Cells were plated in 96 well plates and cultured for 24 hours. Thereafter 10 µl of medium containing various concentrations of compounds were added to the wells and incubated for 48 hours. The final DMSO concentration in all the wells was 0.2 %. After 48 hours, MTT reagent was added and incubated for 4 hour, followed by solubilisation reagent. After 16 hours, the absorbance of the plates was determined at 595 nm and the IC_{50} values were calculated as described in materials and methods.

Generally the ligands appeared to be slightly more active on their own than when complexed to a platinum metal atom (Table 3.6), particularly in the WHCO1, WHCO5 and KYSE150 cell lines. However, ligand 60 which showed the most activity when complexed to a platinum metal (compound 84) showed the least activity in the absence of the platinum metal atom.

The ligands appeared to be less active on their own compared to when they were complexed to a gold metal atom, suggesting that the presence of a gold metal instead of a platinum metal enhanced their activity. Similar observations were made when the ligands were tested on the cervical cancer cell lines and normal fibroblast cells (Figure 3.7).

Table 3.6

Testing the effects of ligands on cell viability

Four ligands were tested for their effects on cell viability by conducting MTT assays on oesophageal cancer cell lines. IC_{50} values that showed ambiguity as seen on Table 3.3 or those that were above 100 µM are expressed as undefined (UD). This result represents a single experiment done in triplicate and data analysis was done on graphpad.

IC_{50} µM (95 % CI)					
LIGANDS	**OESOPHAGEAL CANCER CELL LINES**				
	WHC01	**WHC05**	**KYSE150**	**KYSE410**	**KYSE450**
60	UD	UD	UD	UD	UD
62	36 (29 to 45)	45 (39 to 51)	53 (39 to 72)	UD	UD
63	40 (32 to 49)	65 (36 to 117)	85 (50 to 144)	UD	UD
64	35 (20 to 63)	44 (22 to 87)	23 (13 to 40)	85 (55 to 131)	83 (43 to 159)

The MTT assays were only carried out once for the platinum compounds but because the experiment was conducted on a wide range of cell lines and the results obtained were consistent across all the different cell lines, it was safe to conclude that the platinum compounds were inactive. The gold compounds showed significant amounts of activity and the ligands displayed varying activity ranging from being slightly active to showing no activity. Furthermore the activity displayed by the gold compounds seemed to be ligand independent as compound 95, which lacked the ligand backbone, was observed to have similar activity to compounds 93 and 94.

Table 3.7

Testing the effects of ligands on cell viability

Four ligands were tested for their effects on cell viability by conducting MTT assays on cervical cancer cell lines and normal fibroblast cultures. IC_{50} values that showed ambiguity as indicated on Table 3.3 or those that were above 100 µM are expressed as undefined (UD). This result represents a single experiment done in triplicate and data analysis was done on graphpad.

IC_{50} µM (95 % CI)				
LIGANDS	**CERVICAL CANCER CELL LINES**		**NORMAL FIBROBLAST**	
	CASKI	**HELA**	**DMB**	**FG_0**
60	UD	UD	UD	UD
62	62 (57 to 68)	54 (45 to 65)	UD	51 (46 to 55)
63	UD	89 (77 to 102)	UD	UD
64	UD	76 (58 to 99)	UD	UD

To confirm the activity observed for the gold compounds and the ligands, MTT assays were repeated on the three gold compounds and on ligands 63 and 64, which are specific to compound 93 and 94, respectively. Even though the compounds do not show selectivity in killing cancer cells, it is still important to characterise compounds that may have a mode of action different from that of cisplatin and other compounds that have been associated with multiple side effects. For this reason further experiments were conducted on the gold (I) chloride compounds, which showed the most activity, against a broad range of cells. The experiments that follow were conducted using concentrations obtained after averaging IC_{50} values from the first and second experiments conducted on the gold (I) compounds, where the mean value for compound 93 was 5 µM, compound 94 was 6 µM and 5 µM was obtained for compound 95.

3.3 The effects of gold (I) chloride compounds on cell morphology

From the previous MTT assays it was established that the gold compounds affect cell viability. It was thus interesting to determine whether the effects were associated with any obvious morphological changes. WHCO1 cells were treated with the indicated compounds at 1X and 2X the IC_{50} concentrations (mean of IC_{50} values obtained from two independent MTT experiments) for 12, 24 and 48 hours and the cell morphology was observed at each time point (Figure 3.4). DMSO treated cells were used as a negative control and doxorubicin (dox) treated cells were used as a positive control (Ctrl).

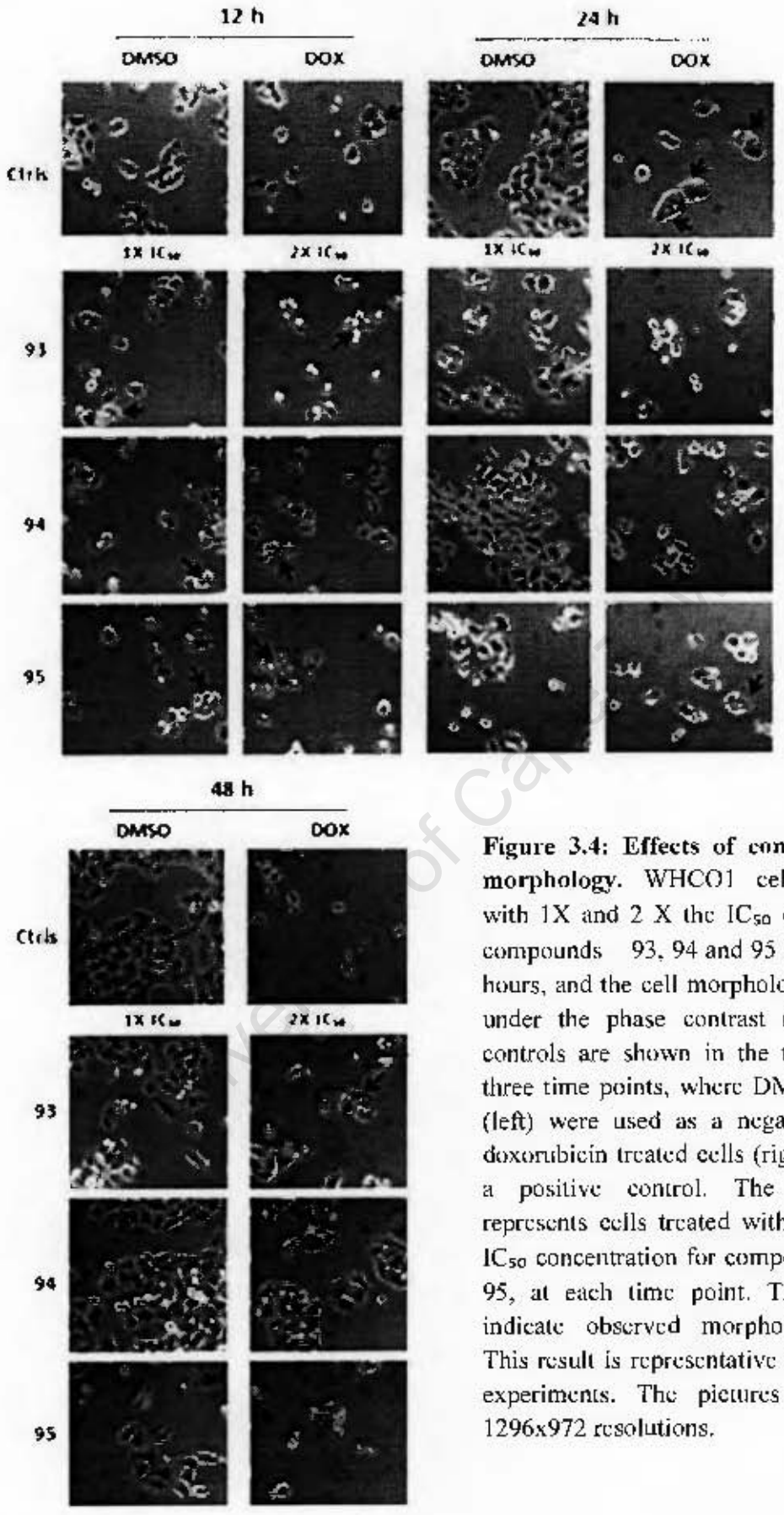


Figure 3.4: Effects of compounds on cell morphology. WHCO1 cells were treated with 1X and 2 X the IC_{50} concentrations of compounds 93, 94 and 95 for 12, 24 and 48 hours, and the cell morphology was observed under the phase contrast microscope. The controls are shown in the top panel for all three time points, where DMSO treated cells (left) were used as a negative control and doxorubicin treated cells (right) were used as a positive control. The bottom panels represents cells treated with 1X and 2X the IC_{50} concentration for compounds 93, 94 and 95, at each time point. The black arrows indicate observed morphological changes. This result is representative of 3 independent experiments. The pictures were taken at 1296x972 resolutions.

After 12 hours of treatment the cells treated with doxorubicin appeared more rounded when compared to DMSO treated cells, similar observations were made for the cells treated with compounds 93, 94 and 95 at 1X and 2X the IC_{50} concentration. In addition to this a decrease in cell number was observed for the cells treated with the indicated compounds when compared to DMSO treated cells (consistent with the MTT results). Cell clumping was observed for the positive control and the cells treated with 10 μM (2X the IC_{50} concentration) of compound 95 at 24 hours. A decrease in cell number was observed for all gold (I) treated cells accept for the cells treated with 1X the IC_{50} concentration (6 μM) of compound 94. Similar to the earlier time points a decrease in cell number was observed for the higher concentrations for all gold (I) compounds and the positive control at 48 hours.

In conclusion, treating cells with compounds 93, 94 and 95 led to a decrease in cell number, particularly in the cells treated with the higher concentrations, when compared to DMSO treated cells, for all time points.

3.4 Time course of effects of compounds on cell viability

One of the hallmarks of cancer as reviewed by Hanahan *et al.* (2000) is the ability of cancer cells to evade anti-growth signals and to provide their own growth signals.[82] This uncontrolled growth of cancer cells is also associated with the invasion of the basement membrane and the spread of the cancer to other tissues via the lymph and blood. Therefore the ability to limit cell proliferation is essential in preventing tumour growth.

Since in the previous section we had shown that the gold (I) compounds were capable of reducing cell number, here we wanted to determine the time course of the effect of these compounds. MTT assays were conducted and the effects of compounds on cell proliferation

were monitored over a period of five days. Compounds 93, 94 and 95 appeared to inhibit cell growth in a time and dose dependent manner (Figure 3.5). Cell growth inhibition was observed 24 hours after treating cells with compounds 93 (10 µM) and 94 (12 µM) and this inhibition was maintained for more than 72 hours. Compound 95 (10 µM) appeared to have noticeable effect on cell growth inhibition 72 hours after treatment.

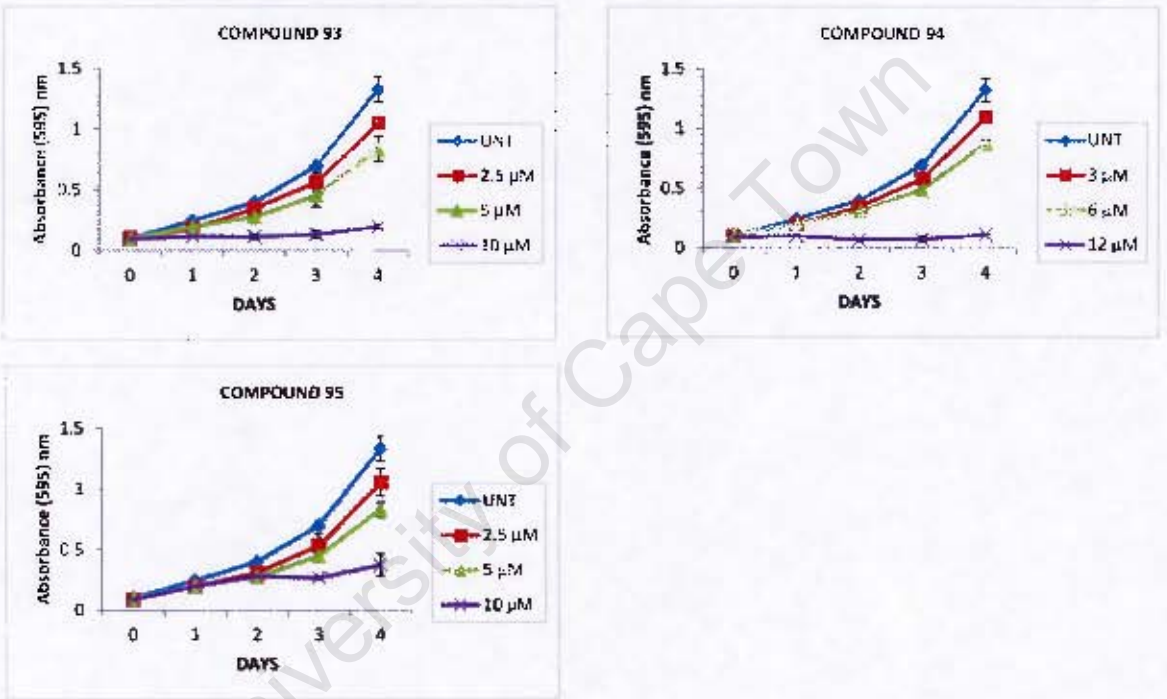


Figure 3.5: Cell growth assay. WHCO1 cells were treated with 0.5, 1X and 2X the IC_{50} concentration as described in the materials and methods before adding MTT and solubilising reagent. These results represent 3 independent experiments conducted in triplicate and the error bars denote the standard deviation (SD).

As it has been mentioned previously, the cell cycle plays an important role in regulating cell growth and part of the screening process during drug discovery involves looking at the effects of novel compounds on cell proliferation. Thus the observations made from the growth assays directed us to investigate the effect of these compounds on the cell cycle.

3.5 Effects of gold (I) chloride compounds on the cell cycle profile

The cell cycle, which is the core machinery for regulating cell proliferation, exhibits checkpoints that amongst other processes monitor DNA integrity, an essential step that identifies DNA mutations and ensures accurate DNA duplication during synthesis.[116] As mentioned above, tumour cells have an ability to evade anti-growth signals and this is commonly associated with defective cell cycle check points. To assess the effects of our compounds on the cell cycle profile, WHCO1 cells were treated with the various compounds for 12, 24 and 48 hours and the cell cycle was monitored. During this experiment doxorubicin was used as a positive control. An example of a typical cell cycle profile obtained after treating WHCO1 cells with the gold compounds is presented in Figure 3.6. However, all the data obtained at each time point was transformed into histograms to facilitate analysis (Figure 3.7).

An increase in the S phase was observed 12 hours after treatment with all three compounds, compared to the DMSO treated cells (Figure 3.7). The same observations were made at 24 and 48 hours, suggesting a time- and dose-dependent inhibition of the cell cycle. These observations were supported by the statistical data shown in Table 3.8, where a student t-test was used to determine the statistical significance of the S phase arrest for the different treatments, by comparing DMSO treated cells to cells treated with compound.

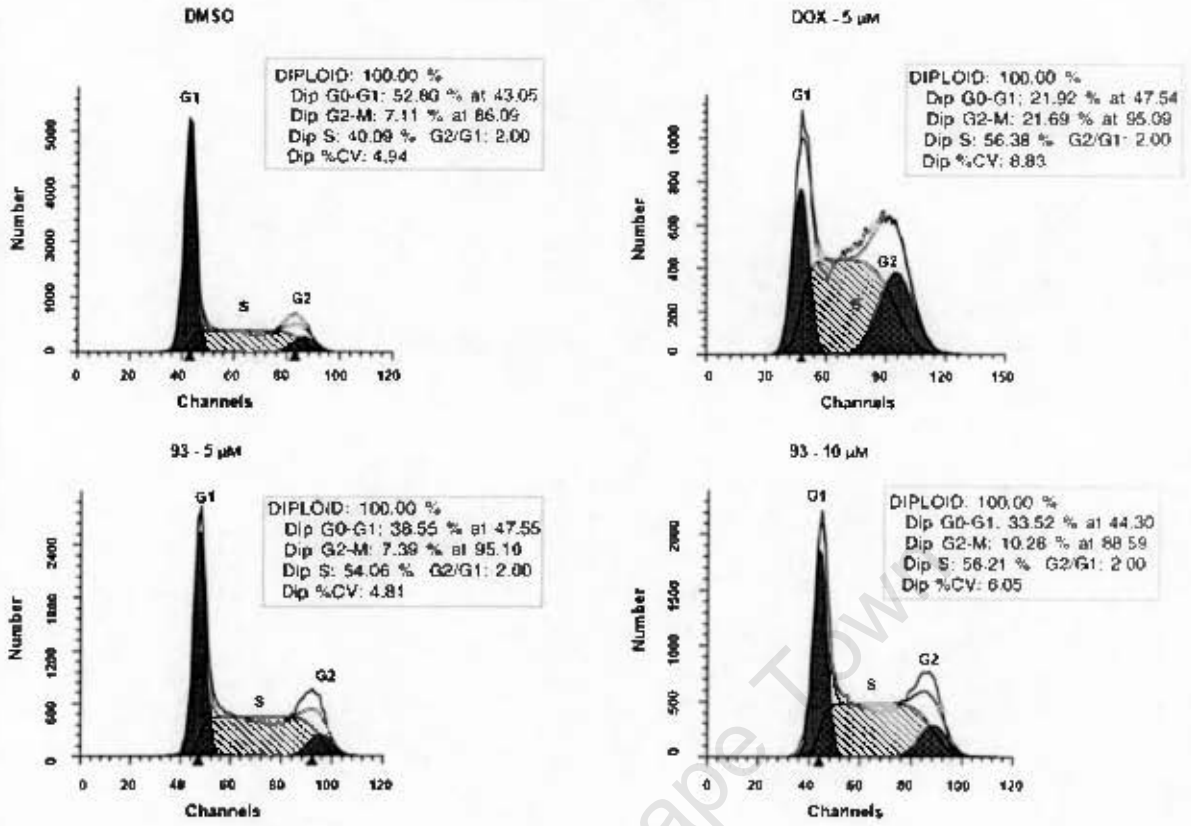


Figure 3.6: Cell cycle profile for gold (I) compounds. This data represents an overview of the results obtained after treating WHCO1 cells with gold (I) compounds. $3x10^5$ WHCO1 cells were plated and incubated overnight at 37°C. The cells were treated with 1X and 2X the IC_{50} concentration for 12, 24 and 48 hours. Cells were harvested at the indicated time points by trypsinisation and the sample were processed as described in materials and methods. Samples were analysed on a Facs calibur using the cell Quest Pro. programme.

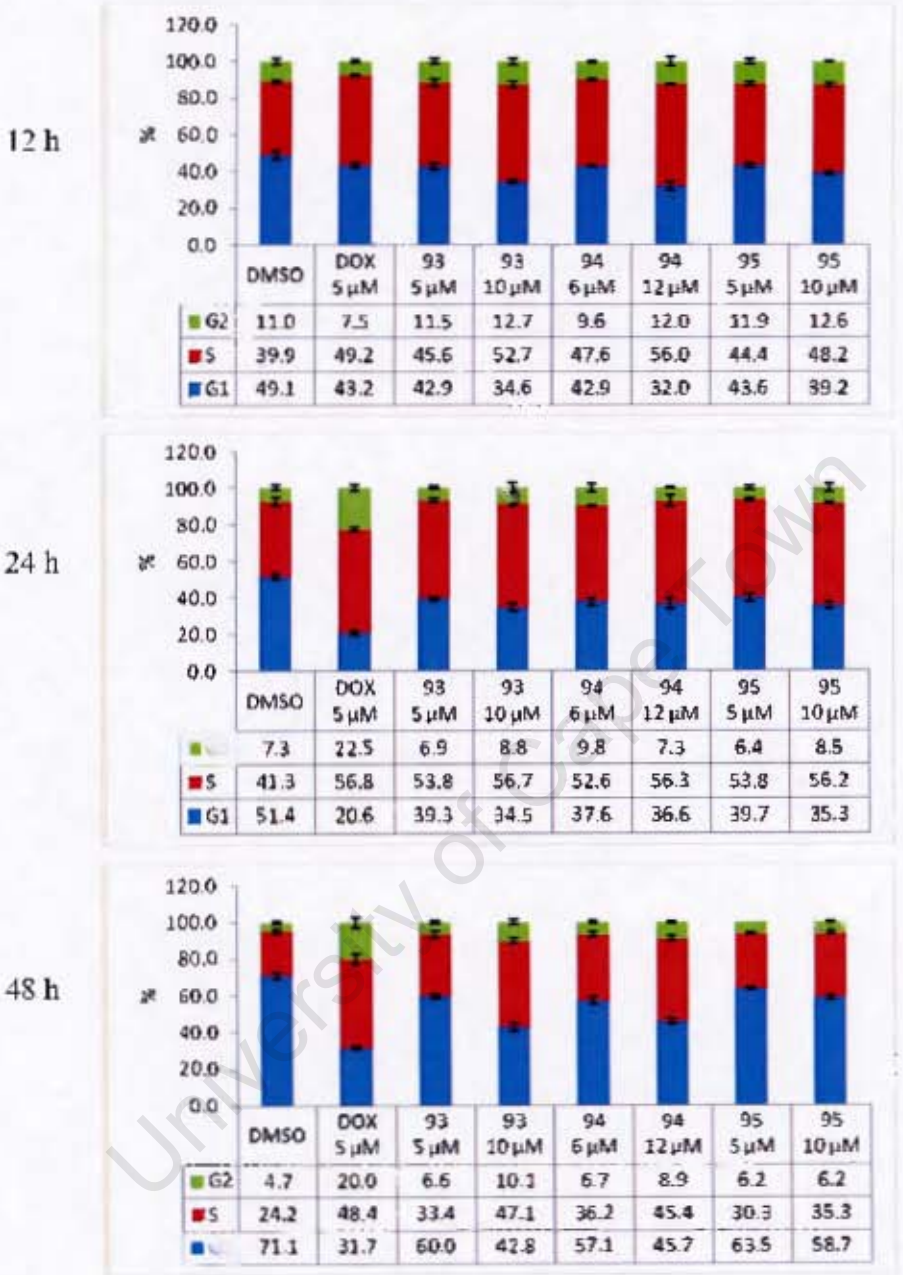


Figure 3.7: Changes observed in the cell cycle profile after treating cells with the gold (I) compounds. WIICO1 cells were treated with 1X and 2 X the IC_{50} concentrations for 12, 24 and 48 hours. Changes in the cell cycle were determined by flow cytometry. Mean values are shown on the tables below the graphs. These results are representative of 3 independent experiments conducted in triplicate. The error bars denote the SD.

Table 3.8

Statistical analysis for cell cycle profile results

Statistical analysis was done on the percentage of cells in the S phase only, where DMSO treated cells were compared to cells treated with doxorubicin and the gold (I) compounds for 12, 24 and 48 hours. These results are representative of three independent experiments conducted in triplicate and the replicates for each treatment condition were used to calculate the p-values using the student t-test (Results obtained from the other two experiments are shown on Tables 5 and 6 in the appendix A). The mean and standard deviation (Stdev) for each treatment point are also included in the table. * indicates significance $p<0.05$

Statistical analysis (TTEST)									
	12 h			24 h			48 h		
	Mean	Stdev	p-value	Mean	Stdev	p-value	Mean	Stdev	p-value
DMSO	39.9	0.8		41.3	2.2		24.2	1.2	
5 µM DOX	49.2	0.4	0.004428*	56.8	1.2	0.012472*	48.4	3.0	0.006920*
5 µM 93	45.6	2	0.032614*	53.8	1.1	0.021125*	33.4	2.1	0.009658*
10 µM 93	52.7	1.7	0.012049*	56.7	1.1	0.001754*	47.1	1.5	0.000232*
6 µM 94	47.6	0.6	0.006138*	52.6	0.3	0.015257*	36.2	1.3	0.014031*
12 µM 94	56	0.3	0.000388*	56.3	3.1	0.005277*	45.4	1.4	0.001219*
5 µM 95	44.4	1.3	0.021956*	53.8	0.7	0.017115*	30.3	0.6	0.027504*
10 µM 95	48.2	0.9	0.014815*	56.2	0.7	0.008854*	35.3	0.7	0.000759*

A significant inhibition (* $p< 0.05$) of the cell cycle at the S phase was observed 12 hours after treatment with the gold (I) compounds, except in the cells treated with compound 93 (5µM) (Table 3.8). The data obtained at the 24 and 48 hour time points was statistically different from the DMSO treated cells (* $p< 0.05$), and this was consistent with the observations made in Figure 3.7.

3.6 Effects of the gold (I) compounds on cell migration

Since we had already monitored the effects of these compounds on cell viability and the cell cycle profile, we also wanted to determine if these compounds had an effect on cell migration, another important characteristic of tumorigenesis. WHCO1 cells were treated with compound as described in the materials and methods and the scratch width was measured at 0, 8 and 24 hours after treatment. The cells were treated with Mitomycin C (MM), an inhibitor of cell proliferation, to ensure that the decrease in scratch width was due to migrating cells and not proliferating cells. At time 0 (Figure 3.8, first panel from the left) it was observed that the scratch widths were approximately the same size. 8 hour after treatment, the scratch width had decreased by ±40 % in the cells treated with DMSO, Mitomycin C, compound 93 and compound 94, where as only a ±20 % decrease was observed in the cells treated with compound 95. At 24 hours, the scratch width for the DMSO treated cells had come to almost a complete close and the MM treated cell remained more or less in the same position as observed at 8 hours. A further 10 % decrease in the scratch width was observed for the cells treated with compound 93, while a ±5 % increase in the scratch width was observed for compound 94 and a similar but opposite effect was observed for compound 95.

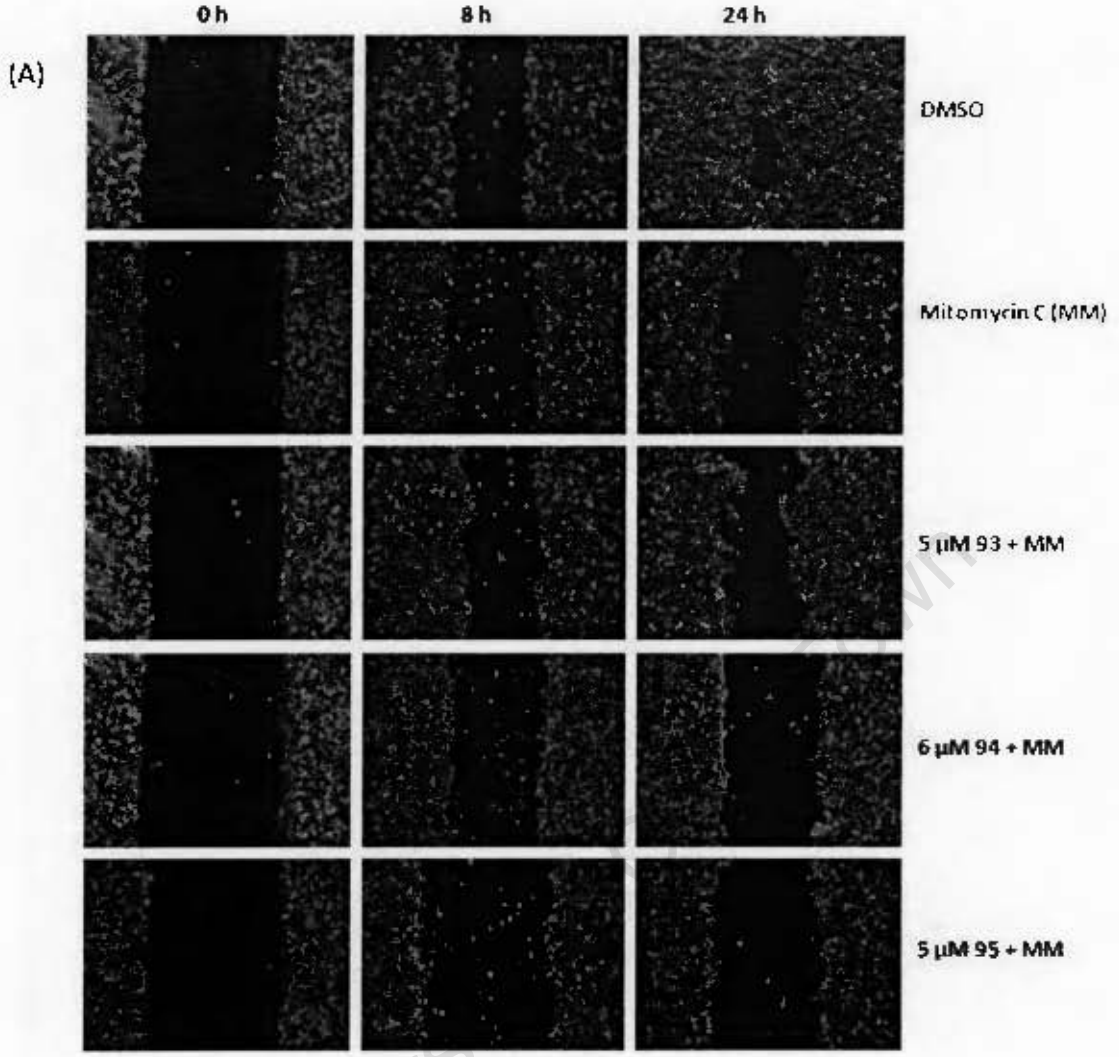


Figure 3.8: Cell migration assay. WHCO1 cells were treated with DMSO only or 10 µg/ml of Mitomycin C (MM) along with either 5 µM of compound 93 or 95 and 6 µM of compound 94. (A) The scratch width was measured at 0, 8 and 24 hours after treatment. (B) The results were quantitated by plotting normalised data on the graph. This result is representative of 3 independent experiments. Two-way ANOVA analysis was performed using Bonferroni posttest, which compared all treated cells to MM treated cells at each time point. $p < 0.0001$ (***), $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*).

3.7 Discussion

Twelve compounds were synthesised in our laboratory and were tested for anticancer activity. These compounds were grouped into four categories which are shown in the schematic diagram below (Figure 3.9). From the cell viability assays, most of the platinum complexes (85, 86, 87 and 88) were inactive against most of the cell lines tested, as well as the fibroblast controls. However, compound 84 appeared to have activity against the cancer cells but was slightly less active against normal fibroblast, suggesting that it would be useful to carryout further investigations on this compound in the future. The gold complexes seemed to be much more active than the platinum complexes and the free ligands, with the order of activity being gold complexes, free ligands and then the platinum complexes, suggesting that the presence of the gold metal atom may contribute to the activity of the complexes. These findings are consistent with the reports in literature. Where cytotoxic and immunoenhancement studies have been conducted on gold complexes and their ligands and it was observed that the gold complexes were more active than their ligands which showed little or no activity.[139,140] A study which was conducted by Marzano *et al.* (2007) compared the activity of two known chemotherapeutic agents, auranofin, a gold (I) compound and cisplatin, which is a platinum (II) compound. In this study the two compounds were tested on a cisplatin sensitive cell line (2008) and a cisplatin resistant cell line (C13*) and auranofin was found to be highly effective, where IC_{50} values less than 1.5 μM were obtained for both cell lines. An IC_{50} value of 6.5 μM was obtained for cisplatin in the 2008 cell line and 114.8 μM was obtained for the C13* cell line.[122] In addition, another study which compared the activity of gold complexes to cisplatin as well as to Tin and Silver complexes, indicated that the gold compounds were more active than cisplatin, and less active when compared to some tin

complexes; however they where found to share the same level of activity with the silver complexes.[78]

All three gold complexes (93, 94 and 95) displayed similar high levels of activity against most of the cell lines tested. This suggested that the chemical and the structural nature of the ligand had very little impact on the activity of the gold complex.

Using WHCO1 cells as our model system, we were able to show that the gold (I) compounds (compound 93, 94 and 95) inhibit cell proliferation in a time- and dose-dependent manner (Figure 3.5). However, the gold (I) chloride compounds were found to be equally active against normal cells, a quality indicative of a certain level of general toxicity. In an article by El-sayyad *et al.* (2009), *in vivo* studies were conducted on cisplatin, doxorubicin and 5-fluorouracil, where all three compounds were found to contribute to skeletal muscle and adipose tissue depletion, suggesting that toxicity is a common thread amongst anticancer therapeutic agents.[129]

Although the gold complexes significantly blocked cell proliferation in a wide panel of cells, they had no apparent effect on cell migration as measured in the wound healing assay. However, compound 95 on the other hand, significantly reduced cell migration (Figure 3.8). Although not shown in gold (I) compounds, there is evidence in the literature to suggest that gold compounds (specifically gold (III) compounds) inhibit cell migration.[45]

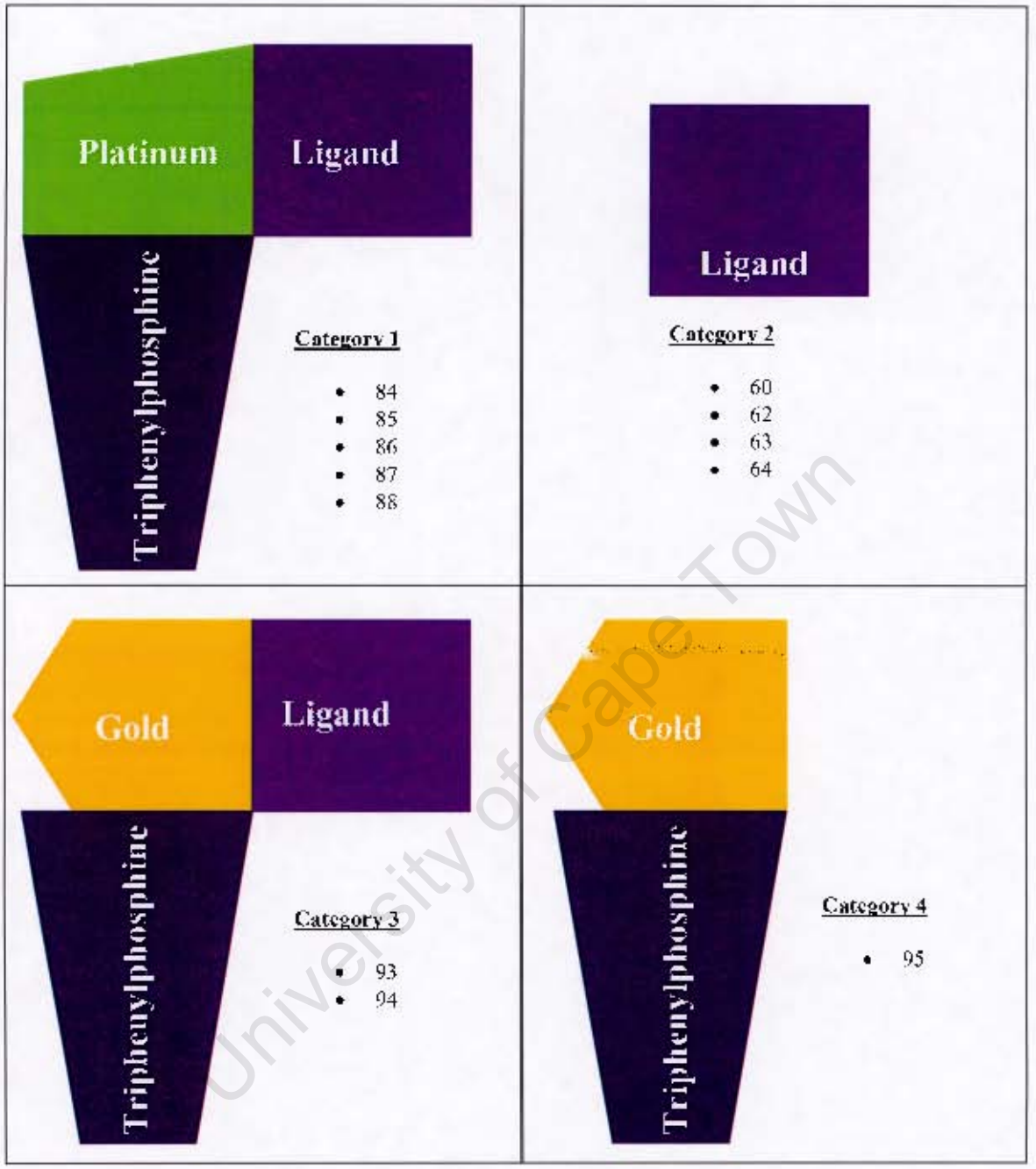


Figure 3.9: A simplistic cartoon of the twelve compounds that were tested for anticancer activity. Five compounds were synthesised from category one, four from category two, two from category 3 and 1 from category four. All twelve compounds shared structural similarities.

A dose dependent S phase arrest was observed 12, 24 and 48 hours after treatment for all three gold (I) compounds, where an increase in the number of cells in the S phase correlated with a decrease in the number of cells in G1 (Figure 3.7). A review article by M. D. Garrett (2001), suggested that cell cycle arrest at the G1/S transition is caused by the presence of double strand breaks (DSBs) in the DNA double helix and the cell cycle arrests at this point to prevent damaged cells from entering into S phase.[141] Normally, when DNA damage is detected by the cell cycle checkpoints, the cell cycle arrests to allow the damaged DNA to be repaired, and if the DNA can not be repaired then apoptosis is initiated. However reports in the literature indicate that when DNA damage is detected at the S phase checkpoint the cell cycle does not arrest but instead DNA replication is slowed down.[141] Based on this evidence we may consider the possibility that treating cells with our compounds may cause DNA damage that is detectable at the G1 checkpoint causing a G1 cell cycle arrest. This speculation correlates with the observations made in Figure 3.7 at the 48h time point, where less cells were observed in the S phase and an increase in cell number at G1, suggesting a G1 cell cycle arrest. Thus, to verify these speculations the presence of DNA damage should be determined.

In summary, based on the observations made in the cell viability assays and the cell cycle profile, compounds 93, 94 and 95 seem to possess cytostatic effects that are both time and concentration dependent.

To further establish the anticancer properties for the gold (I) chloride complexes, experiments to identify the molecular mechanism of action for these compounds, were conducted.

Chapter 4

Effect of gold (I) complexes on selected biological processes

4.1 Introduction

In their review article, Lord and Ashworth (2010) indicated that understanding normal cell function has been a vital tool in the attempt to dismantle the biological defects that lead to tumour development.[142] Furthermore this has led to the identification of several mechanisms that are used by chemotherapeutic agents to inhibit cell proliferation and induce cell death.[142,143]

In the previous section, we had demonstrated that the novel gold (I) complexes (93, 94 and 95) display significant activity against a broad panel of oesophageal cancer cell lines, as well as in the few breast and cervical cancer cell lines tested. Considering the substantial increase in activity of the ligands observed subsequent to complexation with gold, and not platinum, we designed a series of experiments to explore how the gold complexes prevented cancer cell proliferation.

We determined the effect of the gold compounds on: DNA damage (using the phosphorylated H2AX as a marker of DNA damage), induction of apoptosis (by monitoring the cleavage of PARP as a marker of apoptosis), redox systems (by monitoring the thioredoxin reductase system), and on ROS levels (by measuring ROS levels inside cells using a probe sensitive to ROS levels).

4.2 Determining the levels of phosphorylated H2AX after treatment with gold (I) chloride compounds

In the literature, there is evidence to suggest that some metal complexes block the growth of cancer cells, in part by causing DNA damage.[141,144] To assay for DNA damage, phosphorylated H2AX (γH2AX), an indicator of DNA damage, was detected by Western blot analysis. Doxorubicin and DMSO treated cells were used as positive and negative controls, respectively.

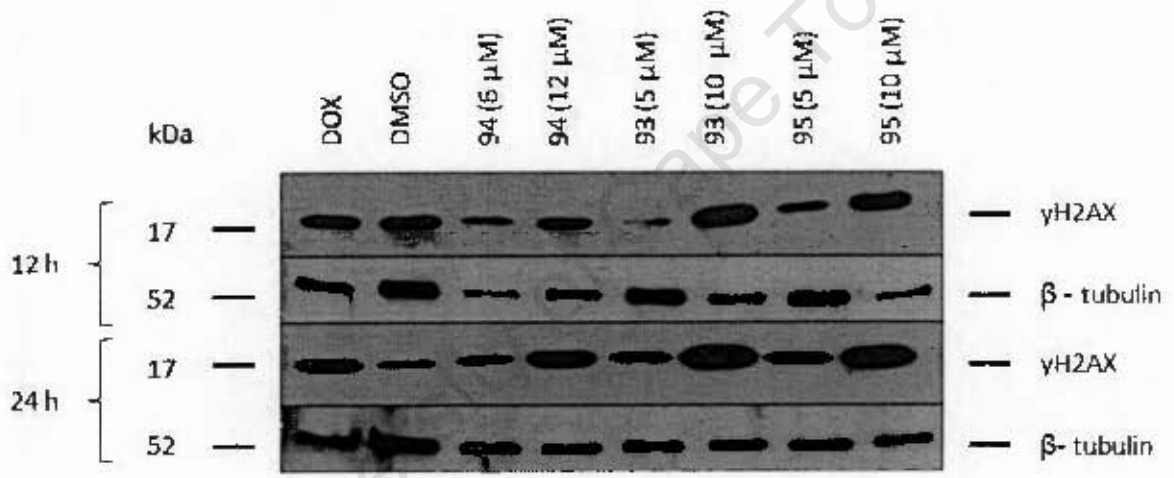


Figure 4.1: Detecting levels of phosphorylated H2AX after treatment with gold (I) compounds. γH2AX levels were detected by Western blot analysis at 12 and 24 after treating the cells with 1X and 2 X the IC_{50} concentrations. Doxorubicin (dox) and the DMSO treated cells were used as positive and negative controls, respectively. This result is representative of three independent experiments.

From Figure 4.1, it appeared as though for the 12 hour time point, phosphorylated H2AX levels were only induced by compound 93 and 95 at 10 μM. However the β-tubulin levels indicated that there was uneven loading. But when comparing β-tubulin levels in the DMSO treated cells to that in the cells treated with compound 93 and 95 (10 μM), the DMSO treated

cells were observed to have higher levels of β-tubulin. This observation suggested that the intense bands which were observed for compounds 93 and 95 (10 μM), were a true representation of the levels of phosphorylated H2AX, as low levels of γH2AX were detected in the DMSO treated cells. When comparing the β-tubulin levels of DMSO treated cells to those of doxorubicin and compound 94 (6 μM), it was evident that the uneven loading may have influenced the outcome of the observation made at these treatment points as low levels of γH2AX seemed to correlate with low levels of β-tubulin and the opposite was true.

In contrast, treatment of cells with the gold (I) compounds for 24 hours resulted in a noticeable increase in H2AX phosphorylation, particularly at the higher concentrations of the compounds. A slight increase in γH2AX was observed for the cells treated with 5 μM of compound 93 and 95 when compared to DMSO treated cells. The β-tubulin levels at this time point seemed even in the cells treated with gold (I) compounds. However higher levels of β-tubulin were observed in the DMSO treated cells, but this did not seem influence the levels of γH2AX.

In summary, the induction of phosphorylated H2AX levels after treatment with gold (I) chloride compounds appeared to be time and concentration dependent. However, further experimentation is required for reproducibility and to solidify the current data.

4.3 Assessing levels of cleaved PARP after treatment with gold (I) chloride compounds

The data in Fig. 4.1 suggests that the gold (I) complexes do induce DNA damage within 24 hours of treatment. Under normal circumstances, if the damaged DNA can not be repaired, the cells are forced into apoptosis.[41,141] To determine if treatment with the gold (I) compounds drives the cells to undergo programmed cell death, levels of cleaved PARP were detected by Western blot analysis. PARP is a nuclear protein that is involved in DNA repair and during apoptosis, PARP is cleaved by caspases 3.[104,105,145,146] Doxorubicin treated cells were used as a positive control and the DMSO treated cells were used as a negative control. At the 12 hour treatment point, a faint band corresponding to cleaved PARP was only detected in the positive control (Figure 4.2). Although the loading was uneven at the 12 hour point based on the intensity of the tubulin bands, this same trend was observed 24 hours after treatment. Cleaved PARP corresponds to 89 kDa, and not the strong band observed at approximately 95 kDa in lane 2 at the 12 hour treatment point. At 24 hours, cleaved PARP was detected at the higher concentrations of 93 and 95, with a very faint band observed for compound 94. An increase in the levels of cleaved PARP were observed at 48 hour in all the treated cells, except in the cells treated with10 μM of compound 93, where the cells could be at a later stage of apoptosis. The results observed suggested that the compounds tested induced apoptosis.

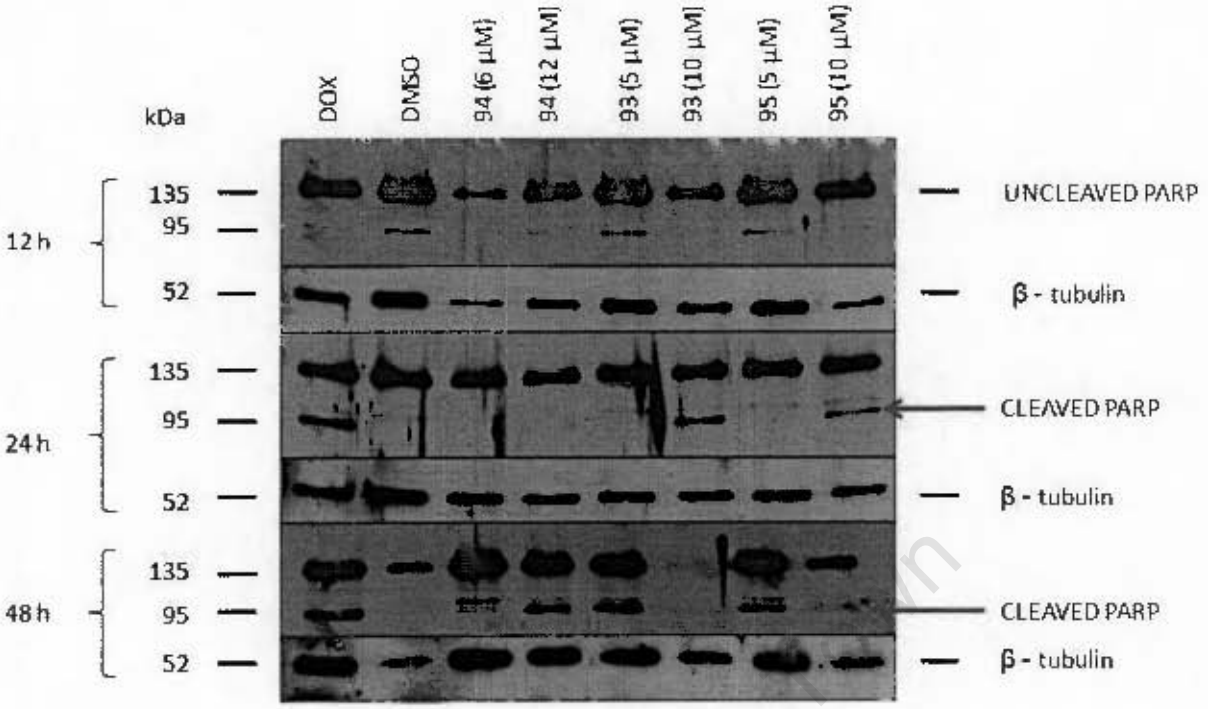


Figure 4.2: PARP detection after treatment with gold (I) compounds. WHCO1 cells were treated as described in materials and methods. Doxorubicin (dox) and DMSO treated cells were used as controls. Uncleaved PARP is 116 kDa and cleaved PARP is 89 kDa. This is representative of three independent experiments.

The process of apoptosis may be triggered through the activation of several pathways, which include the mitochondrial pathway or via the activation of the death receptors (TNFR).[94]

Caruso *et al.* (2007) reported that the phosphine gold (I) compounds induce apoptosis, primarily through targeting the mitochondria of cells.[147] However, other studies have identified auranofin, a gold (I) phosphine drug, as an inhibitor of Thioredoxin Reductase activity in both the mitochondria and the cytoplasm.[122,148,149]

4.4 Determining Thioredoxin Reductase (TrxR) activity in response to treatment with novel gold (I) chloride compounds

The Thioredoxin Reductase system is one of the most important pathways used by cells to regulate their redox status, and this process is NADPH dependent. As shown before (Figure 11), NADPH reduces Thioredoxin Reductase (TrxR), which in turn reduces Thioredoxin (Trx), which then reduces Peroxiredoxin (Prx), which serves as the proximal ROS scavenger.[75,147,148,150] Because compounds 93, 94 and 95 are gold (I) compounds, we thus wanted to determine if, like auranofin, these compounds affect the Thioredoxin Reductase system. Based on the experimental procedures published in most papers, the absorbance for the TrxR assay is most commonly measured at 412 nm and with a few exceptions at 405 nm.[122,138,139,148,151,152] Since the option to measure absorbance at 412 nm was unavailable on our plate reader, but 405 nm was, an assay was conducted to measure the absorbance spectrum of the TrxR assay product across a broad range of wavelengths. No dramatic change in the OD readings was observed between these two wavelengths when we compared the effects of compound 94 on absorbance in the sample treated with compound 94 in the presence and absence of NADPH (Figure 4.3). Therefore the 405 nm wavelength was used to measure the absorbance for the TrxR activity assays.

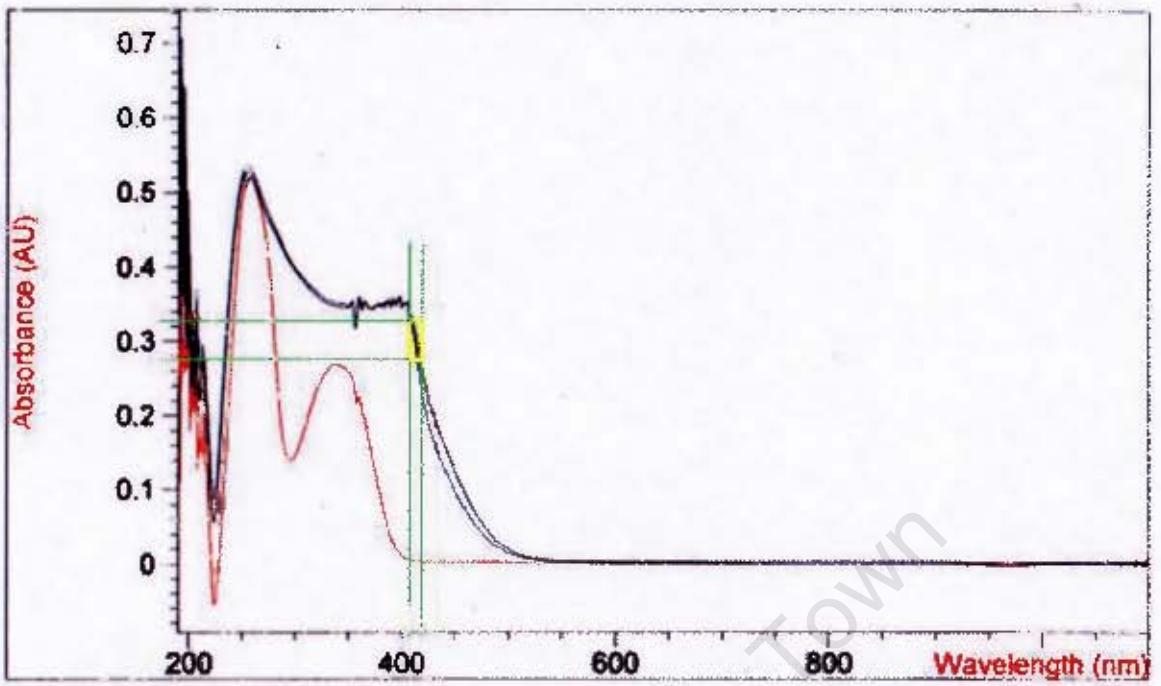


Figure 4.3: Absorbance reading optimisation experiment. Three test tubes were prepared for the Thioredoxin Reductase assay as described in the materials and methods section. Tube 1 (black line) had DMSO treated protein extract plus DTNB and NADPH, tube 2 (blue line) contained a protein extract treated with compound 94 plus DTNB, minus NADPH and tube 3 (red line) protein extract was treated with compound 94 and contained DTNB and NADPH. Each sample was aliquoted into a 0.5 µl cuvette and the change in absorbance at different wavelengths was measured using the UV spectrophotometer. The green vertical lines indicate the 405 and 412 wavelength positions and the green horizontal lines mark the OD values observed at those wavelengths.

Auranofin was used as a positive control for the TrxR assay and before the experiment, an MTT assay was conducted to determine the appropriate concentration of auranofin to be used for the TrxR assay. An IC_{50} value of 3 µM was obtained (Figure 4.4) but, in the literature concentrations of less than 1 µM were used when treating cells with auranofin.[75,148] In our preliminary experiment (Figure 4.5) both 1 µM and 3 µM concentrations were tested for their effects on Thioredoxin Reductase inhibition and were found to have similar levels of inhibition. Therefore the 1 µM auranofin was used as the standard treatment concentration for the rest of the experiments.

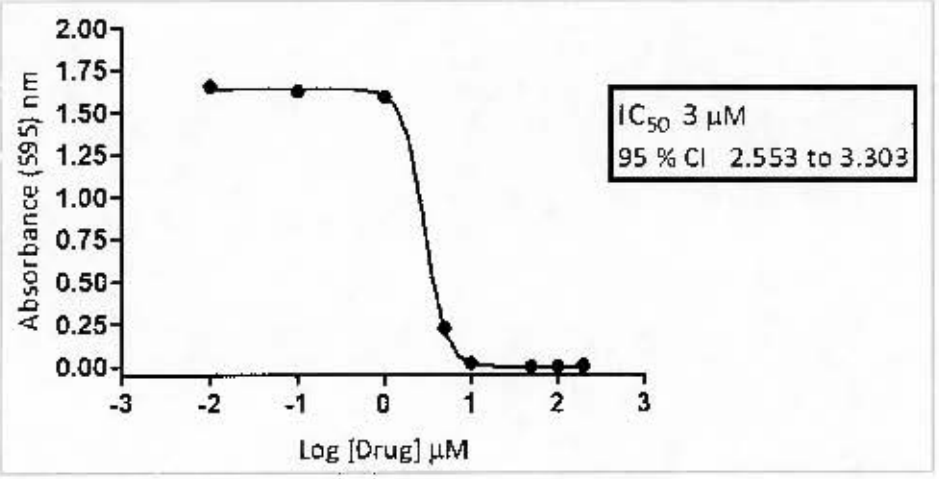


Figure 4.4: IC_{50} concentration for auranofin. An MTT assay was conducted on the WHCO1 cell line to determine the IC_{50} concentration for auranofin. Cells were plated in 96 well plates and cultured for 24 hours. Thereafter 10 µl of medium containing various concentrations of compounds were added to the wells and incubated for 48 hours. The final DMSO concentration in all the wells was 0.2 %. After 48 hours, MTT reagent was added and incubated for 4 hour, followed by solubilisation reagent. After 16 hours, the absorbance of the plates was determined at 595 nm. Data analysis was done on graphpad prism and the data is presented as the mean with error bars represent the SEM. This data is representative of two independent experiments conducted in triplicate.

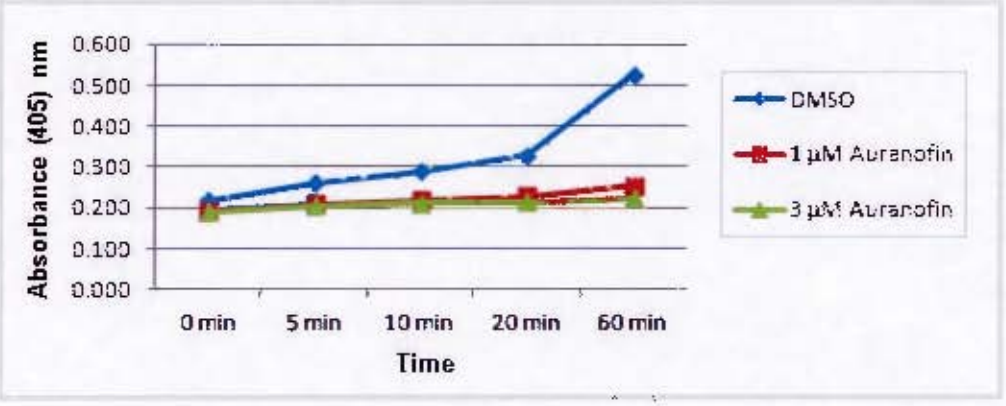


Figure 4.5: Thioredoxin Reductase inhibition by auranofin: The effects of auranofin on Thioredoxin Reductase activity were assessed. 60 µg of total lysate from untreated and treated WHCO1 cells was assayed at the time points indicated. This result is representative of two independent experiments done in duplicate. Mean values were plotted on the graph. The OD values which were obtained from the first and second experiment are shown on Table 7 in the appendix A.

From the preliminary data we were able to determine and confirm the presence of TrxR activity in the WHCO1 model cell line. We were also able to establish that 405 nm could be used to monitor TrxR activity and finally, we were able to confirm that our positive control inhibited the activity of TrxR, as reported previously.

To determine the effects of the novel gold (I) chloride compounds on TrxR activity, WHCO1 cells were treated with compounds 93, 94 and 95 and total TrxR activity was measured. The gold (I) chloride compounds were found to inhibit TrxR activity in a time dependent manner, very similar to that observed for auranofin (Figure 4.6).

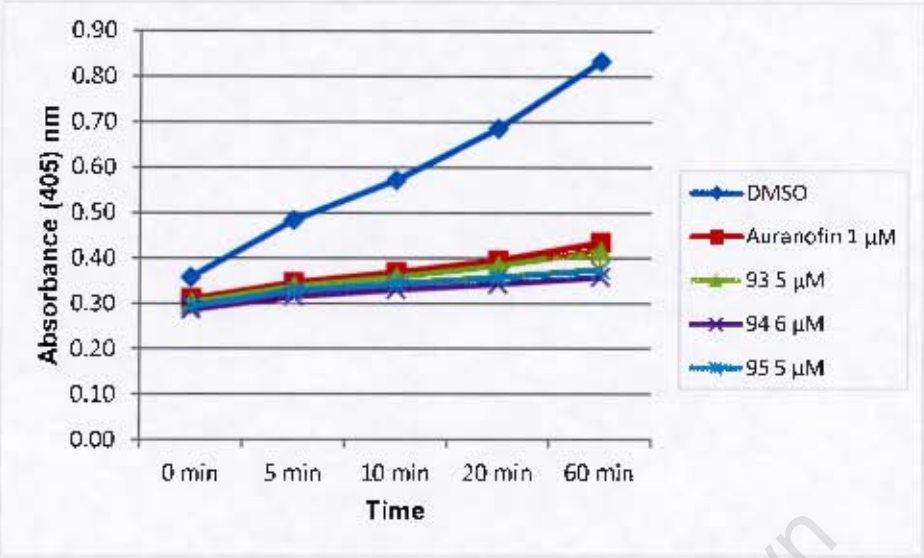


Figure 4.6: Determining the effects of the phosphine gold (I) compounds on total TrxR activity. Total Thioredoxin Reductase (TrxR) was measured (in 60 µg of protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 60 minutes. This result is representative of two independent experiments conducted in duplicate. Mean values were plotted on the graph. The OD values which were obtained from the first and second experiment are shown on Table 8 in the appendix A.

A fractionation assay was conducted to separate the cytoplasmic Thioredoxin Reductase (TrxR1) from the mitochondrial Thioredoxin Reductase (TrxR2) and enzyme activity was then monitored. Similar to the total TrxR inhibition, TrxR1 inhibition appears to be time dependent (Figure 4.7).

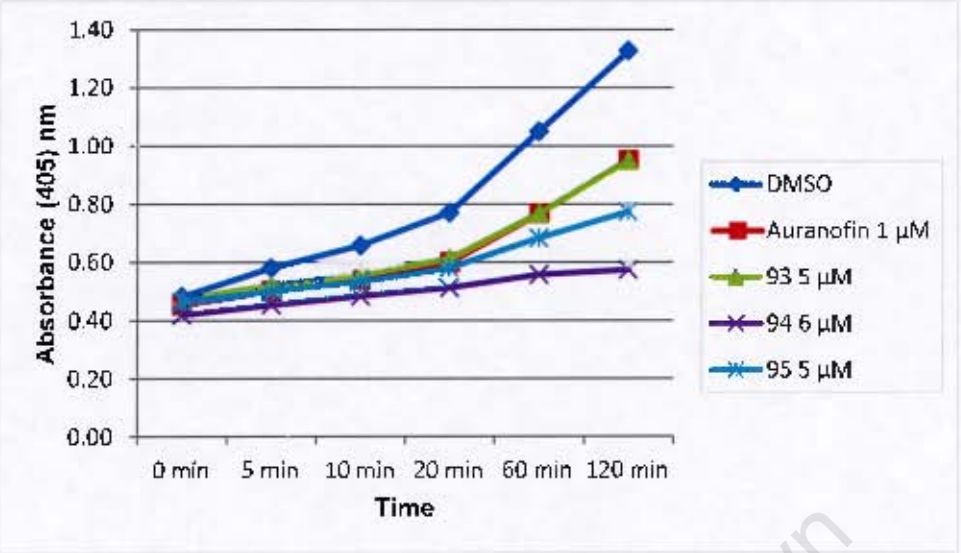


Figure 4.7: TrxR1 inhibition in response to treatment with novel phosphine compounds. Cytoplasmic Thioredoxin Reductase (TrxR1) was measured (in 60 µg of cytoplasmic protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 2 hours. This result is representative of two independent experiments conducted in duplicate. Mean values were plotted on the graph. The OD values which were obtained from the first and second experiment are shown on Table 9 in the appendix A.

All the three compounds that were tested, inhibited TrxR1 (cytoplasmic TrxR) activity to the same extent as auranofin (Figure 4.7). Similar observations were made after measuring TrxR2 (mitochondrial TrxR) activity (Figure 4.8).

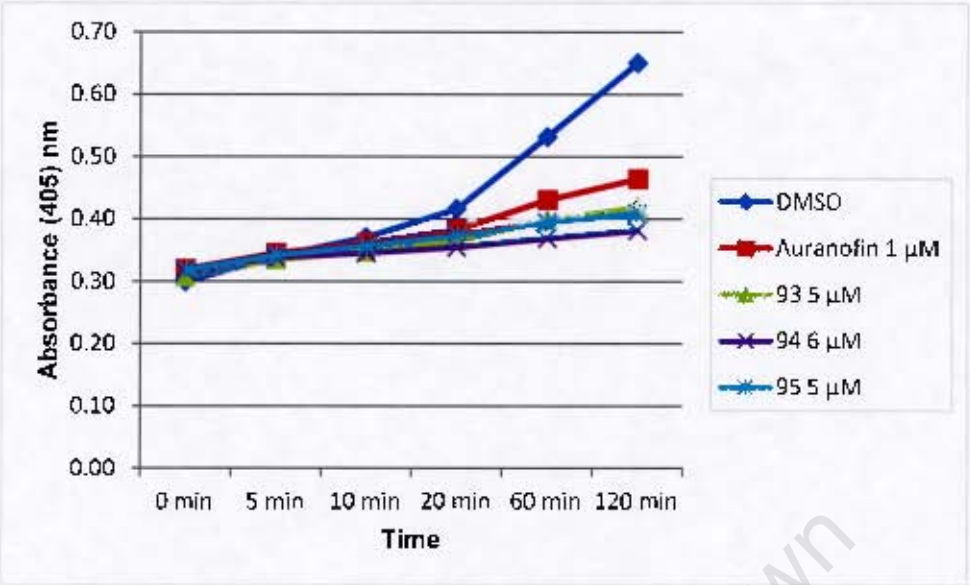


Figure 4.8: TrxR2 inhibition in response to treatment with novel phosphine compounds. Mitochondrial Thioredoxin Reductase (TrxR2) was measured (in 60 µg of mitochondrial protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 2 hours. This result is representative of two independent experiments conducted in duplicate. Mean values were plotted on the graph. The OD values which were obtained from the first and second experiment are shown on Table 10 in the appendix.

4.5 Determining Peroxiredoxin protein levels in response to treatment with auranofin

As was mentioned above, Peroxiredoxin (Prx) is found downstream of TrxR and is reduced by Trx and in its reduced form it acts as a ROS scavenger. From our previous result (Fig. 4.6, 4.7 and 4.8) we showed that treatment of WHCO1 cells with compounds 93, 94 and 95 inhibited TrxR activity. Given this result, we wanted to assess how this inhibition affects the oxidation state of Prx. Auranofin was used as a positive control for the optimisation of the assay.

The protein levels of the three Prx isoforms were determined by Western blot analysis, where the reduction of Prx results in a shift from a dimer to a monomer. Because we had demonstrated that TrxR activity was inhibited by auranofin and our compounds, we anticipated that increased levels of the Prx dimer would be observed in cells treated with auranofin and the gold complexes, relative to untreated cells.

Peroxiredoxin 3 (Prx3), which is found in the mitochondria was monitored to determine its redox state. WHCO1 cells were treated with 1μM and 3 μM of auranofin for 30 minutes, 2 and 4 hours and the protein samples were subject to non-reducing Western blotting conditions. According to the Sigma technical sheet a 31 kDa protein was expected for the monomer. However two very distinct bands of equal intensity were detected and neither of the bands was 31 kDa (Figure 4.9). This experiment was conducted four times under different conditions and the same observations were made under all four conditions. For the first two experiments the primary antibody was dissolved in 2.5 % milk and electrophoresed on a 10 % and 12 % gel. For the other two conditions the primary antibody was dissolved in either 5 % BSA or 5 % milk and subjected to electrophoresis on a 10 % gel. However the same band pattern of equal intensity (as seen on Figure 4.9) was observed under all four conditions.

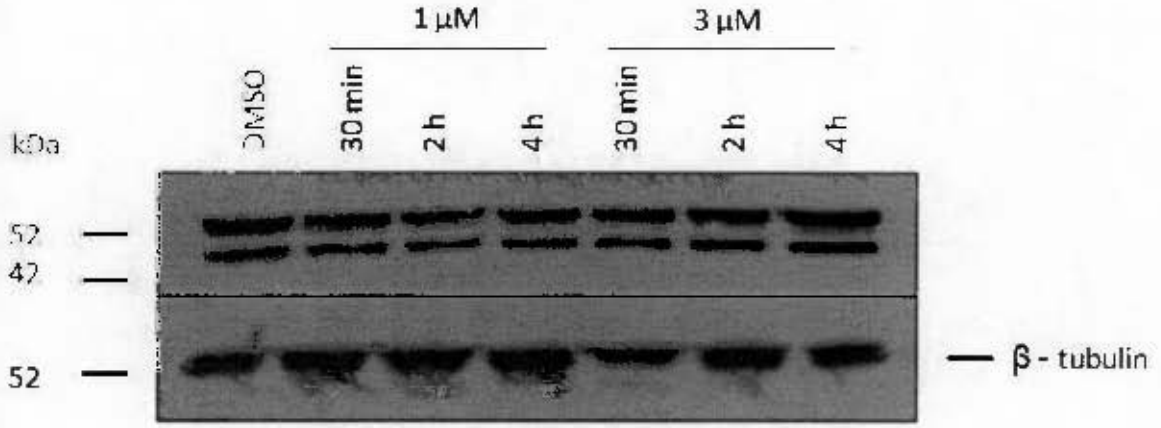


Figure 4.9: Prx3 detection after treatment with auranofin. WHCO1 cells were treated with 1 µM and 3 µM of auranofin for 30 minutes, 2 and 4 hours and 20 µg of protein was loaded onto a gel. The DMSO treated cells were used as a negative control. Double bands (the bottom band is approximately 46 kDa, while the top band is 52 kDa) were detected on the Prx3 blot. This experiment is representative of 4 independent experiments.

The protein levels of the two cytoplasmic Prx isoforms, Prx1 and Prx2 were also determined. According to the Sigma technical sheet, the antibody used to detect Prx1 should detect a 26 kDa monomer. Therefore the dimer or the oxidised form is expected to be approximately 52 kDa. In cells treated with auranofin (1 µM) for various times, no bands corresponding to the expected sizes of Prx1 were observed (Figure 4.10). However, an intense band which could possibly represent the oxidised form of the protein was observed at 42 kDa (assuming that the band detected at 26 kDa is the monomer). However, this seemed unlikely because the same band was detected in the DMSO treated cells (our negative control). Even if we assumed that the proteins were not migrating at the expected molecular weight (since this is a non-reducing gel), there is no clear conversion from monomer to dimer because all the bands at all levels (dimer or monomer) are more or less the same intensity. The results obtained for this experiment were difficult to interpret since we could not be certain that we could unambiguously identify the Prx1 band. There were also a number of non-specific bands

located at the 75 – 95 kDa range that we excluded as possible Prx1 bands because of the size difference.

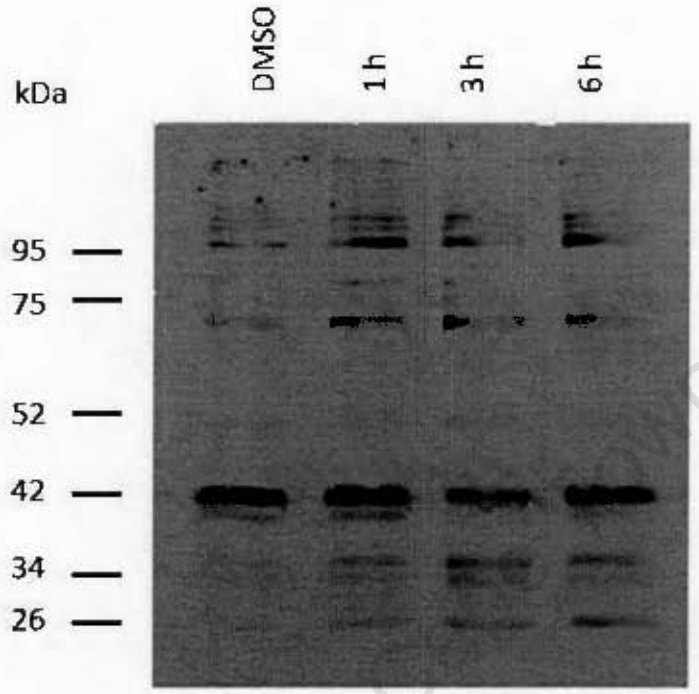


Figure 4.10: Effect of Auranofin on Prx1 levels. WHCO1 cells were treated with 1μM of auranofin for 1, 3 and 6 hours. 20 μg of cell lysate was subjected to non-reducing Western blotting conditions.

A similar experiment was conducted to detect Prx2 in auranofin treated cells. Although a 25 kDa protein (monomer) was expected, many non-specific bands were observed (Figure 4.11). Furthermore, increasing levels of a 34 kDa protein were also observed. The increase in band intensity appeared to be time dependent which was assuring. However because the detected protein size did not correspond to the expected band sizes, there was no way of determining whether the protein detected at 34 kDa was a dimer or a monomer, no conclusion could be drawn from this result. In conclusion none of the Prx proteins were conclusively detected under non-reducing conditions.

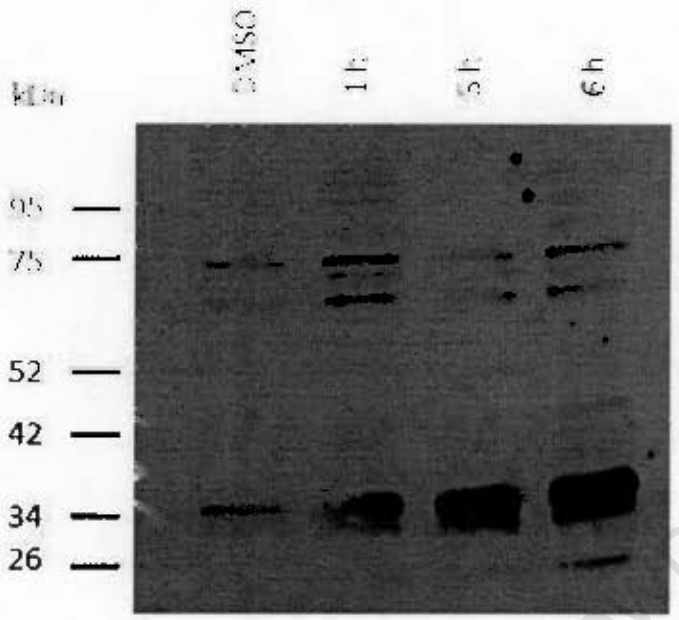


Figure 4.11: Effect of Auranofin on Prx2 levels. WHCO1 cells were treated with 1μM of auranofin for 1, 3 and 6 hours and 20 μg of cell lysate was subjected to non-reducing Western blotting conditions.

4.6 Detecting ROS levels in response to treatment with gold (I) chloride compounds.

The TrxR system regulates ROS levels through the action of Prx and thus if TrxR activity is inhibited (as it has been shown with our WHCO1 cell line), an increase in ROS levels is expected. In order to further define the effects of the novel gold (I) chloride compounds on the Thioredoxin Reductase system, a ROS assay was conducted. As part of the initial optimisation for the experiment, the assay was first conducted on two compounds, doxorubicin (positive control, since we had previously shown in our laboratory that this compound induces ROS production in WHCO1 cells) and compound 93. In Figure 4.12, an increase in ROS production was observed for doxorubicin and compound 93 treated cells when compared to DMSO treated cells. This assay was repeated using high and low concentrations of doxorubicin and compound 93 (Figure 4.13). Similar to the first

experiment, a significant increase in ROS production was observed for both concentrations of doxorubicin. In the cells treated with drug 93, there appeared to be more ROS production at 5 μM than at 10 μM.

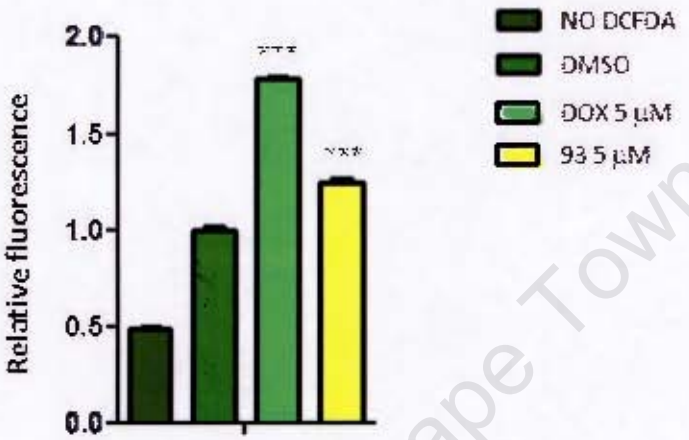


Figure 4.12: Effect of Doxorubicin and Compound 93 on ROS levels. WHCO1 cells were treated with 5 μM of doxorubicin (dox) and compound 93. The ROS assay was carried out as described in materials and methods. NO DCFDA (cells treated with DMSO only), DMSO (cells treated with DMSO in the presence of DCFDA), DOX (positive control). One-way ANOVA analysis was performed using Dunnett's multiple comparison tests, which compares all treated cells to DMSO treated cells. p value < 0.0001 (***).

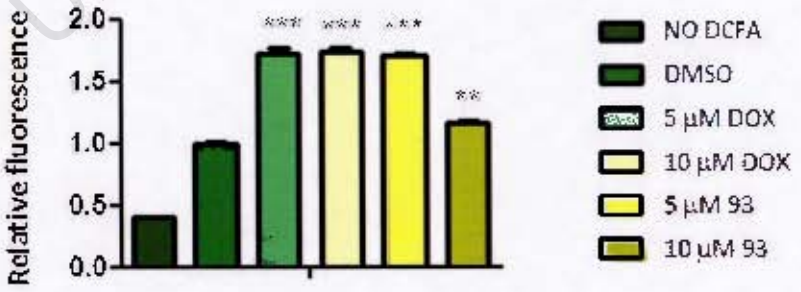


Figure 4.13: Effect of Doxorubicin and Compound 93 on ROS levels. A ROS assay was conducted on WHCO1 cells as described in materials and methods. One-way ANOVA analysis was performed using Dunnett's multiple comparison tests, which compares all treated cells to DMSO treated cells. p value < 0.0001 (***), 0.001< p <0.01 (**).

In summary, we had established that the positive control was working and that treatment with compound 93 induced ROS production. The ROS assay was then conducted on all three gold (I) chloride compounds but an effect opposite to that obtained for the first two experiments was observed. This experiment was repeated several times and the same observations were made on each occasion.

After several unsuccessful attempts at trying to optimise the ROS assay, a different assay approach was developed, where the whole experiment was conducted in 96 well culture plates thus eliminating the excess processing of cells. For this experiment the WHCO1 cells were treated with 1X and 2X the IC_{50} concentrations of compounds 93, 94 and 95. However, no statistically significant increase in ROS production was observed (data not shown). These observations suggested that the ROS reagents may have stopped working and new reagents were required. The ROS assay could not be optimised any further due to time constrains.

4.7 The ROS scavenger assay

Due to the technical difficulties encountered using the ROS assay, it was not possible to conclusively determine whether the gold (I) chloride compounds caused ROS generation, although several experiments suggested this may be the case. Thus to further investigate the involvement of ROS in the response of cells to the gold (I) compounds, the effects of a ROS scavenger was tested. This assay involved monitoring cell number using a crystal violet staining, after treating cells with a combination of various agents for 24 hours. The crystal violet assay was performed to measure cell number, since we observed that the ROS scavengers interfered with the MTT assay. From the results it is clear that treatment with compounds 93, 94 and 95 reduced the number of cells (Figure 4.14) as observed before in the MTT assays. The effects of the gold compounds were less pronounced in the presence of the ROS scavenger N-Acetylcysteine (NAC). A concentration-dependent effect of the ROS scavenger was observed, suggesting that the decrease in cell number correlates with ROS generation.

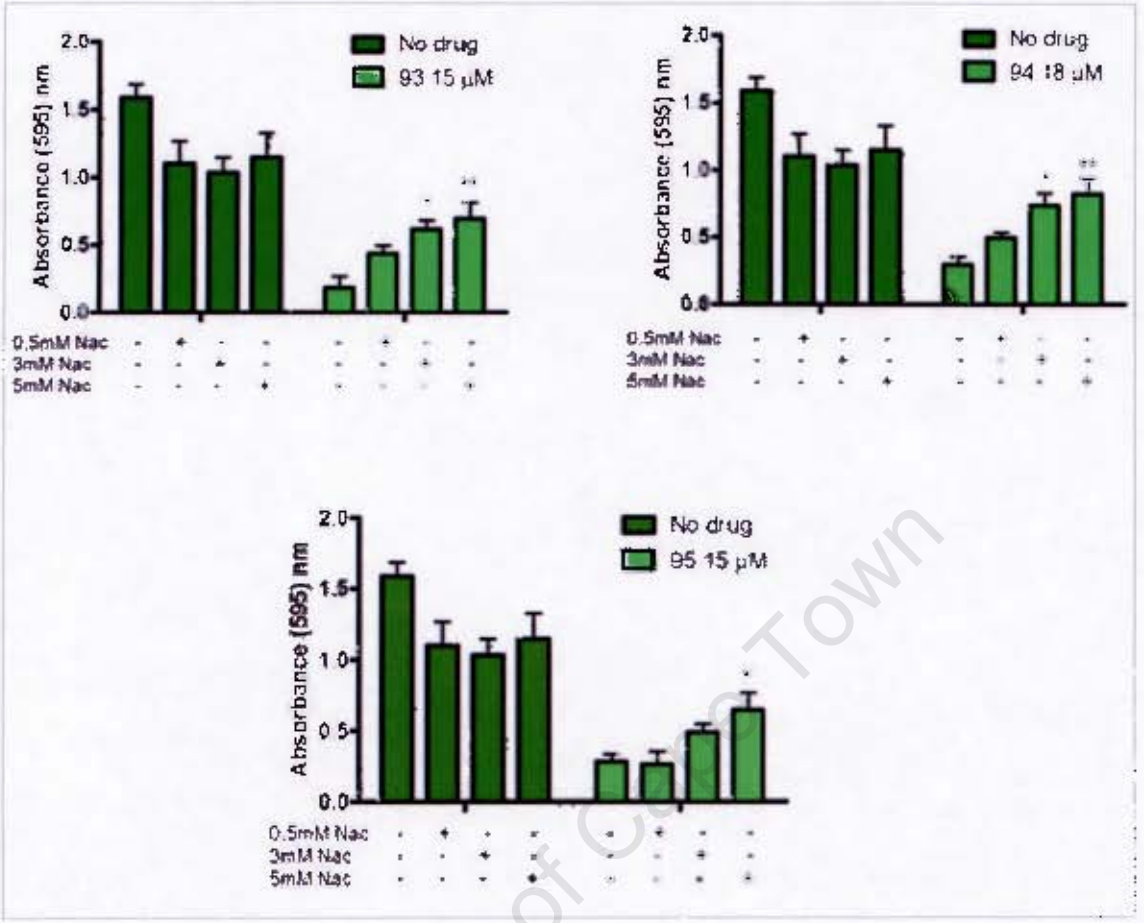


Figure 4.14: ROS scavenger assay. The WHCO1 cell line was treated with 3X the IC_{50} concentrations for 24 hours in the presence and absence of ROS scavenger NAC. Cell number was determined using the crystal violet stain as described in materials and methods. This result is representative of two independent experiments conducted in triplicate. One-way ANOVA analysis was performed using Dunnett's multiple comparison tests, which compares all treated cells to DMSO treated cells (no drug). $0.001 < p < 0.01$ (**), $0.01 < p < 0.05$ (*).

4.8 Discussion

In the previous section (Chapter 3), we had demonstrated that the gold compounds reproducibly blocked the proliferation of a wide range of cancer cells (and normal fibroblasts) at low concentrations. This was associated with a disruption of the cell cycle profile, with a significant increase in the S-phase of the cell cycle after 12, 24 and 48 hours of treatment. In this section we observed that treatment of WHCO1 cells with the gold (I) chloride compounds was associated with DNA damage (visualised as increased levels of phosphorylated H2AX) at 24 hours after treatment for all compounds tested, particularly at the higher concentrations of the compounds. At 12 hours, DNA damage was observed for the cells treated with 10 μM of compounds 93 and 95 (Figure 4.1). It has been suggested that the presence of phosphorylated H2AX is a result of DNA damage caused by DSB.[141] In addition, treatment with the gold (I) compounds also resulted in the induction of apoptosis (visualised as PARP cleavage), 24 and 48 hours after treatment (Figure 4.2). Other studies conducted on gold compounds such as auranofin and Benzimidazol-2-ylidene gold (I) complexes, have demonstrated that gold (I) compounds induce their anticancer activity through the activation of apoptosis.[122,139]

In previous studies, the growth inhibitory effects of auranofin (another Gold (I) complex) in cancer cells were associated with a significant impairment of the Thioredoxin Reductase (TrxR) activity in treated cells.[75,138,139] As mentioned previously, the TrxR system in cells forms part of an important defensive network that protects cells against oxidative stress, and damaging this system, could lead to cell death.[111,122,148] Although our plate reader could only measure the absorbance at 405 nm and not 412 as recommended for the TrxR assay[75,148], a scan of the absorbance spectrum of the TrxR assay product suggested that measuring the absorbance at 405 nm, would not affect our ability to monitor TrxR activity (Fig. 4.3).

Auranofin was used as a positive control during the TrxR activity assay, and before the assay was performed, an MTT assay was conducted to determine the IC_{50} value for auranofin. In most studies, IC_{50} values less than 1 μM have been obtained after treating other cell lines (for example leukemia culture cells) with auranofin[74,148], however an IC_{50} value of 3 μM was obtained for the WHCO1 oesophageal cancer cell line, suggesting that the sensitivity of auranofin may be cell line specific. This is not unexpected. In our experimental system we observed that auranofin depleted TrxR activity in the oesophageal cancer cells as reported in the literature for ovarian, lung, osteosarcoma, cervical, luekemia and liver cancer cultured cells.[75,122,148,153] Furthermore, similar to auranofin, our gold compounds also severely inhibited TrxR activity in WHCO1 cells, reducing total, cytoplasmic and mitochondrial TrxR activity levels (Figure 4.6, 4.7 and 4.8). This suggests that the gold compounds (93, 94 and 95) depleted TrxR activity inside the cells, potentially exposing cells to oxidative stress. However, we were unable to show increased levels of the oxidised form of the peroxiredoxin proteins in cells treated with Auranofin as reported in the literature. Factors such as drug degradation, protein degradation, the possibility that the buffers and stock solution were not prepared properly as well as problems with protein harvesting, were considered during troubleshooting. However the possibility of drug degradation was ruled out as other experiments that were conducted using the same drug stocks were successful, furthermore treating the cells with fresh stocks of auranofin was not successful. New buffer and stock solutions were made up, freshly extracted protein was used in some cases and the assay protocol was modified several times, yet the experiment was not successful. We were unable to show reproducible elevation of ROS levels in cells treated with the gold complexes, although our results were very suggestive for compound 93 (Figure 4.12 and 4.13). In contrast, the ROS scavenger, NAC consistently, partially protected the cells from cytotoxicity caused by treatment with compounds 93, 94 and 95 (Figure 4.14). Although this protection

was not complete, these results strongly suggested that the reduction in cell number caused by the gold (I) compounds was mediated in part through ROS. As mentioned previously, there is evidence in the literature to suggest that gold (I) compounds induce apoptosis by inhibiting TrxR activity and this is associated with an up-regulation of ROS.[122,138,139] In a study conducted on a novel gold (I) compound [Au(d2pypp)2]Cl, bis[1,3-bis(di-2-pyridylphosphino)propane] gold(I) Chloride, the authors demonstrated that this compound induced apoptosis via caspase activation which was associated with the depleted membrane permeability transition (MPT) and the loss of the glutathione (GSH) pool, as a result of compound built up in the mitochondria. Furthermore, the authors indicated that the TrxR system is essential for maintaining the redox state in the cells and that compound [Au(d2pypp)2]Cl inhibited TrxR activity causing mitochondrial dysfunction, leading to the activation of apoptosis.[138] In addition, a study conducted on auranofin suggested that this gold (I) compound induced apoptosis through the alteration of pro- and anti- apoptotic protein levels from the Bcl-2 family, rather than via the MPT pore.[148] Here we have demonstrated that our compounds inhibit thioredoxin activity and that they seem to induce apoptosis 24 hours after treatment, as well as that they may potentially have an effect on ROS. Thus for future work, it may be useful to design experiments to investigate how these compounds affect the MPT pore, caspase activity and pro- and anti-apoptotic proteins. It will also be useful to determine the anticancer properties of these compounds on animal models, as the above experiments were only conducted in cultured cells.

Chapter 5

Effects of gold (I) compounds on CXCR2 and IGF1R knockdown cells

5.1 Introduction

Cell surface receptor that mediate cell proliferation, survival and cell migration signals such as epidermal growth factor receptor (EGFR), Insulin-like growth factor -1 receptor (IGFR) and Interleukin 8 receptor (CXCR2) are over expressed in many cancers, including oesophageal cancer.[80,82,154–157] There are many reports in the literature that show that these receptor systems play an important role in contributing to the survival and proliferation of cancer cells (over normal cells).[136,158,159] There are a number of studies that have been conducted which demonstrate that these receptor are valid targets in the treatment of some cancers.[136,157,158] In our laboratory, we have also shown that CXCR2 overexpression contributes to oesophageal cancer survival.[160] Since CXCR2 and IGF1R are thought to contribute survival signals to cancer cells, we wanted to determine if oesophageal cancer cells in which CXCR2 and IGF1R had been knocked down with shRNA, displayed increased sensitivity to the gold compounds compared to the wild type cells.

5.2 Results

Using IGF1R and CXCR2 stable knockdown cells that were established in our laboratory, cell viability assays were conducted to determine the effects of phosphine gold (I) compounds on these cell lines. The presence of knockdown was confirmed by western blot analysis where reduced levels of IGF1R and CXCR2 protein levels were detected in the WHCO1 and WHCO6 (result provided by Dr Luke Esau) knockdown cell lines, compared to cells where scrambled shRNA had been transfected (Ctrl shRNA) (Figure 5.1 and 5.2).

After stable knockdown had been confirmed, MTT assays were conducted on the WHCO1 parental cells, WHCO1 Control (Ctrl) shRNA cells, WHCO1 CXCR2 shRNA knockdown cells and WHCO1 IGF1R shRNA knockdown cells, as well as on the WHCO6 parental cells and respective knockdown cells.

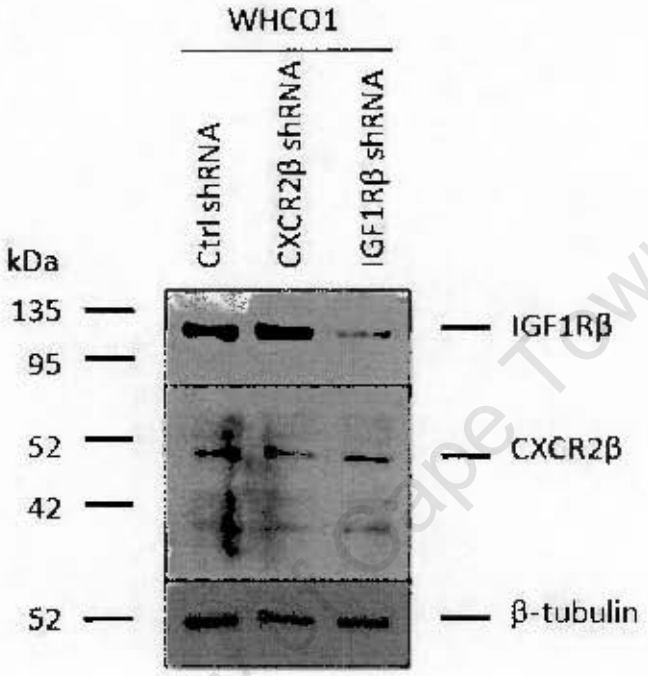


Figure 5.1: CXCR2 and IGF1R stable knockdown in the WHCO1 cell line. Interleukin 8 receptor (CXCR2) beta and Insulin-like growth factor -1 receptor (IGF1R) beta knockdown was determined in the WHCO1 cell line. 20 µg of protein was electrophoresed.

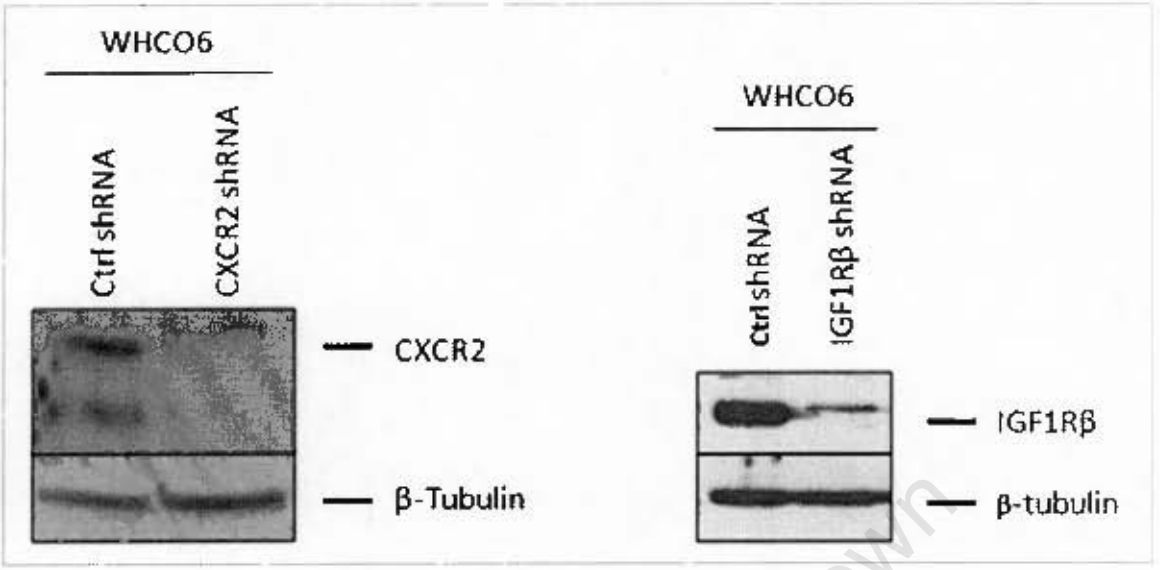


Figure 5.2: CXCR2 and IGF1R stable knockdown in the WHCO6 cell line. This result was obtained from a PhD student in our laboratory. Interleukin 8 receptor (CXCR2) beta and Insulin-like growth factor -1 receptor (IGF1R) beta knockdown was determined in the WHCO6 cell line.

A slight decrease in the IC_{50} concentration was observed in the CXCR2 knockdown cells; however this decrease was not significant because the 95 % CI of the control and knockdown cells overlap (Table 5.1a). The same observations were made for the CXCR2 knockdown cell lines treated with compound 94 (Table 5.1b) and compound 95 (Table 5.1c).

An IC_{50} concentration of 15.59 μM was observed for the WHCO1 cells treated with compound 93 (Table 5.2a), and in the IGF1R knockdown cells, an increase in the IC_{50} concentration was observed for both the WHCO1 and WHCO6 knockdown cell when compared to the control cells. The same observations were made for the cells treated with compound 94 (Table 5.2b) and compound 95 (Table 5.2c).

In principle treating the knockdown cells with the phosphine gold (I) compounds was hypothesized to cause either a synergistic or additive effect, where knockdown of the receptors would reduce the cancerous phenotype of the cells and treatment with the chemotherapeutic agent would thus caused the cells to be more susceptible to apoptosis. However this was not the case as seen with our data.

Table 5.1

WHCO1 and WHCO6 CXCR2 shRNA knockdown cells treated with phosphine gold (I) compounds. MTT assays were conducted on WHCO1 and WHCO6 parental cells, Control (Ctrl) shRNA cells, and CXCR2 shRNA knockdown cells. Tables show IC_{50} values obtained after treatment with (a) compound 93, (b) compound 94, (c) and compound 95. These results are representative of two independent experiments conducted in triplicate.

Compound 93

Cell line	IC_{50} (μM)	95 % CI
WHCO1	12.34	10.51 to 14.49
WHCO1 Ctrl shRNA	13.07	11.05 to 15.46
WHCO1 CXCR2β shRNA	11.31	9.05 to 14.12
WHCO6	12.37	10.24 to 14.96
WHCO6 Ctrl shRNA	14.08	9.48 to 20.91
WHCO6 CXCR2β shRNA	11.63	8.70 to 15.54

Compound 94

Cell line	IC_{50} (μM)	95 % CI
WHCO1	9.54	8.46 to 10.77
WHCO1 Ctrl shRNA	10.40	9.73 to 11.12
WHCO1 CXCR2β shRNA	9.28	8.72 to 9.87
WHCO6	10.58	9.03 to 12.40
WHCO6 Ctrl shRNA	11.50	10.23 to 12.93
WHCO6 CXCR2β shRNA	11.80	10.21 to 13.64

Compound 95

Cell line	IC_{50} (μM)	95 % CI
WHCO1	6.99	5.27 to 9.28
WHCO1 Ctrl shRNA	9.59	7.92 to 11.61
WHCO1 CXCR2β shRNA	8.48	7.52 to 9.55
WHCO6	6.91	4.54 to 10.50
WHCO6 Ctrl shRNA	10.75	9.57 to 12.08
WHCO6 CXCR2β shRNA	11.31	9.65 to 13.24

Table 5.2

WHCO1 and WHCO6 IGF1R shRNA knockdown cells treated with phosphine gold (I) compounds. MTT assays were conducted on WHCO1 and WHCO6 parental cells, Control (Ctrl) shRNA cells, and IGF1R shRNA knockdown cells. Tables show IC_{50} values obtained after treatment with (a) compound 93, (b) compound 94, (c) and compound 95. These results are representative of two independent experiments conducted in triplicate.

Compound 95

Cell line	IC_{50} (μM)	95 % CI
WHCO1	15.59	10.04 to 24.21
WHCO1 Ctrl shRNA	8.16	7.35 to 9.07
WHCO1 IGF1Rβ shRNA	12.80	9.24 to 17.73
WHCO6	8.22	7.64 to 8.85
WHCO6 Ctrl shRNA	9.49	7.95 to 11.34
WHCO6 IGF1Rβ shRNA	16.16	12.87 to 20.28

Compound 94

Cell line	IC_{50} (μM)	95 % CI
WHCO1	10.35	9.43 to 11.37
WHCO1 Ctrl shRNA	7.41	6.96 to 7.89
WHCO1 IGF1Rβ shRNA	8.57	7.65 to 9.61
WHCO6	8.87	7.88 to 9.99
WHCO6 Ctrl shRNA	8.96	8.23 to 9.75
WHCO6 IGF1Rβ shRNA	10.93	9.99 to 11.97

Compound 95

Cell line	IC_{50} (μM)	95 % CI
WHCO1	12.17	10.23 to 14.49
WHCO1 Ctrl shRNA	8.48	7.13 to 10.08
WHCO1 IGF1Rβ shRNA	9.37	8.54 to 10.29
WHCO6	10.78	9.26 to 12.54
WHCO6 Ctrl shRNA	9.83	8.56 to 11.28
WHCO6 IGF1Rβ shRNA	11.34	9.96 to 12.91

5.3 Discussion

When CXCR2 and IGF1R knockdown cells were combined with novel compounds, no improvement in efficacy was observed. Thus we can conclude that combination treatment with compound 93, 94 and 95 did not lead to any synergistic or additive effect instead the compounds were observed to be slightly antagonistic.

Conclusion

In their review article, Urig and Becker (2006) highlighted several Thioredoxin reductase (TrxR) inhibitors that have been identified and found to be cytotoxic against a wide range of cancers.[161] Here we have identified three potential novel inhibitors of TrxR, which could prove to be therapeutically beneficial in the treatment of squamous cell carcinoma of the oesophagus. In the same review, the authors summarised various ways in which TrxR inhibitors could induce their effects and these included, TrxR inhibition, a decrease in tumour volume, an increase in ROS production, cell cycle inhibition, the induction of apoptosis and many others.[161] In this study we have shown that the novel gold (I) chloride compounds 93, 94 and 95 inhibit cell growth in a time- and dose-dependent manner (Figure 3.2). Although we were unable to show that all three compounds induce ROS production, the presence of ROS was strongly suggested for all three compounds using the ROS scavenger assay (Figure 4.15), where recovery in cell number was observed upon treatment with increasing concentrations of NAC. From analysing the cell cycle profile we showed that treatment with the gold (I) compounds causes a G1 cell cycle arrest (Figure 3.3). Furthermore phosphorylated H2AX, an indicator of DNA damage, was detected after treatment with compounds 93, 94 and 95 (Figure 4.1). By conducting a PARP assay (Figure 4.2), we were able to deduce that the gold (I) compounds induce apoptosis in a time- and dose-dependent manner. Despite the non-selective killing effect observed for these compounds, the wide spectrum of anticancer properties they present points to their potential as therapeutic agents. However, it would appear that these novel compounds do not present any advantages over well know chemotherapeutic agents like auranofin.

Prospective work

To further explore the relationship between ROS production and the inhibition of Thioredoxin reductase activity. The Peroxiredoxin oxidation state should be investigated by Western blot analysis using new antibodies, preferably those that have been used in publication that look at Peroxiredoxin oxidation state using non-reducing Western blotting conditions.[126,148] Furthermore additional optimisation is required for the ROS assay to conclusively establish the effects of compounds 93, 94 and 95 on ROS levels.

Attempts to detect phosphorylated H2AX at earlier time points (2 – 4 hours) using the FOCI assay which quantitates levels of phosphorylated H2AX foci caused by DSBs, were made. Although a slight increase in the number of foci was observed in the treated cells when compared to DMSO treated cells, this data could not be quantitated because the foci appeared in clusters and thus individual foci were indistinguishable. Further more some cells had more staining than other and thus quantitation could have led to obscure and subjective results. The data was analysed by immunofluorescence. However some publications have shown that this assay could be conducted by flow cytometry thus further optimisation of this assay by flow cytometry could be attempted. This would allow for a more sensitive detection of DNA damage at early time points.

Cyclins and cyclin dependent kinases (CDK) that are involved in the G1/S cell cycle arrest (for example, CDK2/cyclin A for S phase and CDK2/cyclin E for G1) should be detected by Western blot analysis to assay for any changes in protein levels, to characterise the deregulation in the cell cycle that was observed.

To support the data obtained from the PARP assay and to confirm that the compounds do induce apoptosis, an independent assay like the Caspase 3/7 activity assay should be conducted.

Experiments to determine the cancer growth inhibitory properties of these compounds were conducted in cultured cells and therefore for future work, the anticancer properties of the compounds should be investigated in animal models.

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Appendix A: Tables and Protein Marker

Table 1 General treatment table: The following treatment table was used to treat cells with compound 93, 5-fluorouracil, auranofin, novel platinum compounds and ligands. All compounds were dissolved in DMSO to make 100mM stock solutions which were stored at -20°C. 5-fluorouracil and auranofin were stored in the dark.

	[1x] µM	[10x] µM	Preparation of 10x pre-stock		
A	0	0	98µl DMEM	2µl DMSO	No drug
B	0.1	1	98µl DMEM	1µl DMSO	1µl (0.1mM drug dilution)
C	1	10	98µl DMEM	1µl DMSO	1µl (1mM drug dilution)
D	5	50	98µl DMEM	1.5µl DMSO	0.5µl (10mM drug dilution)
E	10	100	98µl DMEM	1µl DMSO	1µl (10mM drug dilution)
F	50	500	98µl DMEM	1.5µl DMSO	0.5µl (100mM stock)
G	100	1000	98µl DMEM	1µl DMSO	1µl (100mM stock)
H	200	2000	98µl DMEM	No DMSO	2µl (100mM stock)

Table 2 Treatment table for compound 94 and 95: 50mM stock solutions of compound 94 and 95 were made up in DMSO and stored at -20°C. The following treatment table was used during MTT assays.

	[1x] µM	[10x] µM	Preparation of 10x pre-stock		
A	0	0	98µl DMEM	2µl DMSO	No drug
B	0.1	1	98µl DMEM	1µl DMSO	1µl (0.1mM drug dilution)
C	1	10	98µl DMEM	1µl DMSO	1µl (1mM drug dilution)
D	5	50	98µl DMEM	1.5µl DMSO	0.5µl (10mM drug dilution)
E	10	100	98µl DMEM	1µl DMSO	1µl (10mM drug dilution)
F	25	250	98µl DMEM	1.5µl DMSO	0.5µl (50mM stock)
G	50	500	98µl DMEM	1µl DMSO	1µl (50mM stock)
H	100	1000	98µl DMEM	No DMSO	2µl (50mM stock)

Table 3 Treatment table for Doxorubicin: The following treatment table was used to treat cells with Doxorubicin (Dox) during MTT assays. 50mM stock solution of Dox was made up in dH_2O and stored in the dark at -80°C.

	[1x] µM	[10x] µM	Preparation of 10x pre-stock		
A	0	0	98µl DMEM	2µl DMSO	No drug
B	0.1	1	98µl DMEM	1µl DMSO	1µl (0.1mM drug dilution)
C	0.5	5	98µl DMEM	1.5µl DMSO	0.5µl (1mM drug dilution)
D	1	10	98µl DMEM	1µl DMSO	1µl (1mM drug dilution)
E	5	50	98µl DMEM	1.5µl DMSO	0.5µl (10mM drug dilution)
F	10	100	98µl DMEM	1µl DMSO	1µl (10mM stock)
G	20	200	98µl DMEM	No DMSO	2µl (10mM stock)
H	50	500	98µl DMEM	1µl DMSO	1µl (50mM stock)

Table 4

Cisplatin treatment table

The following treatment table was used to treat cells with cisplatin for MTT assays. 1.6mM stock solution of cisplatin was made up in 1XPBS and stored at 4°C.

			Preparation of 10X pre-stock	
	[1x] µM	[10x] µM	1.6mM stock (µl)	DMEM (µl)
A	0	0	0	100
B	0.5	5	0.6	99.4
C	1	10	1.25	98.75
D	10	100	6.2	93.8
E	15	150	9.4	90.6
F	30	300	18.8	81.2
G	60	600	37.5	62.5
H	150	1500	93.8	6.2

Table 5

Statistical analysis for cell cycle profile results (first experiment)

Statistical analysis was done on the S phase only, where DMSO treated cells were compared to cells treated with doxorubicin and the gold (I) compounds for 12, 24 and 48 hours. This experiment was conducted in triplicate and the replicas for each treatment condition were used to calculate the p-values using the student t-test. The Mean and standard deviation (Stdev) for each treatment point are also included in the table. * indicates significance $p<0.05$

Statistical analysis (TTEST)									
	12 h			24 h			48 h		
	Mean	Stdev	p-value	Mean	Stdev	p-value	Mean	Stdev	p-value
DMSO	40.1	10.4		45.6	6.6		26.1	2.4	
5 µM 93	51.6	2.0	0.147554	72.4	2.9	0.035416*	24.0	17.4	0.864328
10 µM 93	73.0	3.2	0.048552*	66.9	2.0	0.041173*	58.2	0.9	0.002153*
6 µM 94	47.7	9.8	0.527653	74.0	1.0	0.018726*	38.4	13.3	0.221287
12 µM 94	62.4	1.7	0.048341*	80.2	13.4	0.055831	67.4	1.3	0.001212*
5 µM 95	35.9	2.7	0.583247	46.6	0.7	0.803895	41.8	6.5	0.063509
10 µM 95	43.6	1.5	0.696802	63.1	15.4	0.295424	43.4	7.3	0.092236

Table 6

Statistical analysis for cell cycle profile results (second experiment)

Statistical analysis was done on the S phase only, where DMSO treated cells were compared to cells treated with doxorubicin and the gold (I) compounds for 12, 24 and 48 hours. This experiment was conducted in triplicate and the replicas for each treatment condition were used to calculate the p-values using the student t-test. The Mean and standard deviation (Stdev) for each treatment point are also included in the table. * indicates significance $p<0.05$

Statistical analysis (TTEST)									
	12 h			24 h			48 h		
	Mean	Stdev	p-value	Mean	Stdev	p-value	Mean	Stdev	p-value
DMSO	38.2	3.9		53.6	1.3		25.1	0.7	
5 µM DOX	55.9	11.1	0.158280	70.9	1.3	0.001429*	66.6	1.4	0.000111*
5 µM 93	70.3	0.2	0.004282*	71.4	1.3	0.001041*	37.1	0.9	0.001457*
10 µM 93	66.4	1.4	0.008634*	78.2	1.3	0.002333*	69.3	1.6	0.000876*
6 µM 94	49.6	0.6	0.043345*	73.2	2.7	0.001815*	48.1	1.9	0.002264*
12 µM 94	67.4	0.5	0.007419*	77.5	8.2	0.031636*	71.7	1.7	0.000461*
5 µM 95	62.4	9.3	0.075162	59.5	7.0	0.257145	28.7	3.5	0.271957
10 µM 95	68.6	3.5	0.001879*	84.9	3.1	0.003865*	51.5	2.1	0.002700*

Table 7

Thioredoxin Reductase inhibition by auranofin

The effects of auranofin on Thioredoxin Reductase activity were assessed on the WHCO1 cell line as described in the materials and methods. This result is representative of two independent experiments conducted in duplicate (Experiment 1 is highlighted in black and experiment 2 in green).

	0 min			5 min			10 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.217	0.22	0.219	0.261	0.262	0.262	0.29	0.291	0.291
	0.436	0.452	0.44	0.534	0.545	0.54	0.606	0.618	0.61
Auranofin 1 µM	0.193	0.193	0.193	0.208	0.21	0.209	0.217	0.219	0.218
	0.396	0.42	0.4	0.427	0.446	0.44	0.446	0.468	0.46
Auranofin 3 µM	0.19	0.19	0.190	0.204	0.205	0.205	0.21	0.212	0.211
	0.377	0.378	0.38	0.405	0.407	0.41	0.422	0.423	0.42
	20 min			60 min					
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average			
DMSO	0.329	0.328	0.3285	0.527	0.526	0.5265			
	0.723	0.737	0.73	1.023	1.039	1.03			
Auranofin 1 µM	0.227	0.229	0.228	0.252	0.256	0.254			
	0.479	0.505	0.49	0.564	0.597	0.58			
Auranofin 3 µM	0.211	0.215	0.213	0.221	0.225	0.223			
	0.447	0.449	0.45	0.494	0.496	0.50			

Table 8

Determining the effects of the phosphine gold (I) compounds on total TrxR activity. Total Thioredoxin Reductase (TrxR) was measured (in 60 µg of protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 60 minutes. This result is representative of two independent experiments conducted in duplicate (Experiment 1 is highlighted in black and experiment 2 in green).

	0 min			5 min			10 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.352	0.365	0.36	0.48	0.488	0.48	0.57	0.575	0.57
	0.3505	0.3625	0.3565	0.476	0.4975	0.48675	0.573	0.6055	0.58925
Auranofin 1 µM	0.313	0.311	0.31	0.348	0.347	0.35	0.37	0.37	0.37
	0.22	0.222	0.22	0.25	0.252	0.25	0.268	0.271	0.27
93 5 µM	0.3	0.307	0.30	0.334	0.343	0.34	0.354	0.365	0.36
	0.212	0.215	0.21	0.238	0.247	0.24	0.253	0.258	0.26
94 6 µM	0.286	0.289	0.29	0.315	0.318	0.32	0.33	0.333	0.33
	0.355	0.361	0.358	0.385	0.393	0.389	0.398	0.406	0.402
95 5 µM	0.294	0.297	0.30	0.327	0.331	0.33	0.342	0.348	0.35
	0.207	0.21	0.21	0.248	0.252	0.25	0.254	0.256	0.26
	20 min			60 min					
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average			
DMSO	0.683	0.689	0.69	0.827	0.841	0.83			
	0.712	0.7635	0.73775	1.069	1.169	1.119			
Auranofin 1 µM	0.396	0.396	0.40	0.435	0.436	0.44			
	0.291	0.294	0.29	0.343	0.346	0.34			
93 5 µM	0.378	0.39	0.38	0.41	0.424	0.42			
	0.27	0.276	0.27	0.303	0.309	0.31			
94 6 µM	0.342	0.346	0.34	0.357	0.361	0.36			
	0.409	0.418	0.4135	0.428	0.436	0.432			
95 5 µM	0.355	0.362	0.36	0.371	0.379	0.38			
	0.262	0.27	0.27	0.294	0.298	0.39			

Table 9

TrxR1 inhibition in response to treatment with novel phosphine compounds. Cytoplasmic Thioredoxin Reductase (TrxR1) was measured (in 60 µg of cytoplasmic protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 2 hours. This result is representative of two independent experiments conducted in duplicate (Experiment 1 is highlighted in black and experiment 2 in green).

	0 min			5 min			10 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.48	0.485	0.48	0.578	0.585	0.58	0.653	0.664	0.66
	0.503	0.517	0.51	0.573	0.592	0.58	0.633	0.648	0.64
Auranofin 1 µM	0.451	0.456	0.45	0.5	0.506	0.50	0.538	0.544	0.54
	0.46	0.478	0.47	0.517	0.543	0.53	0.551	0.581	0.57
93 5 µM	0.466	0.481	0.47	0.514	0.527	0.52	0.549	0.565	0.56
	0.437	0.446	0.44	0.477	0.486	0.48	0.506	0.519	0.51
94 6 µM	0.435	0.403	0.42	0.472	0.436	0.45	0.512	0.456	0.48
	0.398	0.396	0.40	0.438	0.42	0.42	0.43	0.433	0.43
95 5 µM	0.453	0.475	0.46	0.486	0.515	0.50	0.515	0.553	0.53
	0.427	0.439	0.43	0.464	0.479	0.47	0.493	0.508	0.50
	20 min			60 min			120 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.76	0.782	0.77	1.025	1.075	1.05	1.295	1.359	1.33
	0.725	0.713	0.72	0.95	0.911	0.93	1.221	1.172	1.20
Auranofin 1 µM	0.597	0.605	0.60	0.762	0.774	0.77	0.945	0.959	0.95
	0.632	0.636	0.62	0.771	0.816	0.79	0.998	1.052	1.03
93 5 µM	0.604	0.623	0.61	0.758	0.781	0.77	0.936	0.965	0.95
	0.558	0.571	0.57	0.686	0.712	0.70	0.802	0.831	0.82
94 6 µM	0.545	0.481	0.51	0.594	0.522	0.56	0.604	0.544	0.57
	0.443	0.449	0.45	0.462	0.471	0.47	0.478	0.486	0.48
95 5 µM	0.558	0.604	0.58	0.656	0.711	0.68	0.735	0.813	0.77
	0.533	0.552	0.54	0.609	0.641	0.63	0.656	0.677	0.67

Table 10

TrxR2 inhibition in response to treatment with novel phosphine compounds. Mitochondrial Thioredoxin Reductase (TrxR2) was measured (in 60 µg of mitochondrial protein lysate) as described in materials and methods. Enzyme activity was measured at different time intervals over a period of 2 hours. This result is representative of two independent experiments conducted in duplicate (Experiment 1 is highlighted in black and experiment 2 in green).

	0 min			5 min			10 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.307	0.292	0.30	0.346	0.334	0.34	0.376	0.366	0.37
	0.319	0.326	0.32	0.348	0.361	0.35	0.37	0.384	0.38
Auranofin 1 µM	0.324	0.316	0.32	0.348	0.343	0.35	0.364	0.359	0.36
	0.338	0.342	0.34	0.364	0.37	0.37	0.381	0.386	0.38
93 5 µM	0.303	0.313	0.31	0.328	0.342	0.34	0.341	0.354	0.35
	0.282	0.28	0.28	0.296	0.294	0.30	0.303	0.301	0.30
94 6 µM	0.308	0.325	0.32	0.331	0.346	0.34	0.337	0.354	0.35
	0.319	0.324	0.32	0.332	0.335	0.33	0.339	0.343	0.34
95 5 µM	0.317	0.321	0.32	0.34	0.346	0.34	0.354	0.361	0.36
	0.294	0.3	0.30	0.311	0.315	0.31	0.319	0.325	0.32
	20 min			60 min			120 min		
	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average	OD (1)	OD (2)	Average
DMSO	0.421	0.412	0.42	0.536	0.528	0.53	0.654	0.646	0.65
	0.399	0.413	0.41	0.472	0.49	0.48	0.524	0.543	0.53
Auranofin 1 µM	0.386	0.38	0.38	0.435	0.427	0.43	0.469	0.46	0.46
	0.405	0.408	0.41	0.452	0.452	0.45	0.464	0.48	0.47
93 5 µM	0.358	0.372	0.37	0.391	0.405	0.40	0.414	0.424	0.42
	0.314	0.314	0.31	0.335	0.333	0.33	0.334	0.334	0.33
94 6 µM	0.346	0.364	0.36	0.36	0.378	0.37	0.373	0.391	0.38
	0.349	0.355	0.35	0.372	0.373	0.37	0.375	0.374	0.37
95 5 µM	0.371	0.377	0.37	0.392	0.398	0.40	0.407	0.412	0.41
	0.333	0.338	0.34	0.349	0.354	0.35	0.351	0.359	0.36

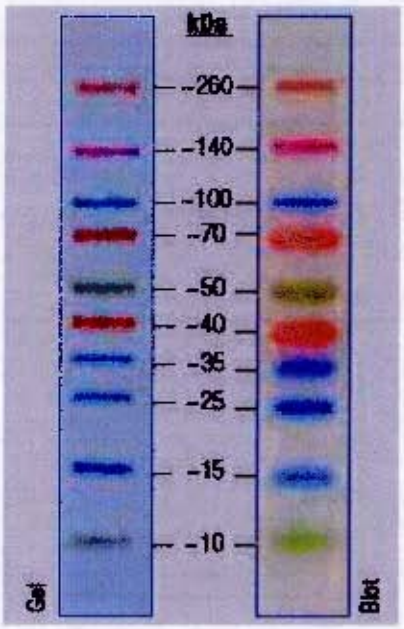

Figure 1: Spectra™ Multicolour Broad Range Protein Ladder (# SM1841). This protein ladder was used in Western blot analysis and it is suitable for detecting proteins of 10 to 260 kDa on 4 % to 20 % gels.

Appendix B: Solutions

B.1 Tissue culture

10x PBS 1L

80g NaCl
2g KCl
11.5g $Na_2HPO_4 \cdot 7H_2O$
2g KH_2PO_4
Make up to 1L with dH_2O
Store at room temperature and at 4°C once diluted to 1x PBS

Heat Inactivated Foetal Calf Serum (FCS)

Heat FCS for 30 minutes at 56°C
Store at -20°C for long term storage and at 4°C once opened

Penicillin / streptomycin solution

Add 5 million units of Penicillin G Sodium (Highveld Biological) to 5ml PBS
Add 5g 214S Streptomycin Sulphate (Highveld Biological) to 15ml PBS
Combine the two solutions and make up to 500ml with PBS
Store at -20°C

Complete DMEM

450ml DMEM
50ml FCS
5ml Penicillin and Streptomycin (P/S)
Store at 4°C

Trypsin-EDTA

0.5g trypsin
8g NaCl
1.45g $Na_2HPO_4 \cdot 2H_2O$
0.2g KCl
0.2g KH_2PO_4
10mM EDTA (pH 8)
Make up to 1L with PBS
Store at -20°C for long term and at 4°C once in use

Mycoplasm staining solution

0.5µg/ml Hoeschst No. 33258 diluted in Hanks Buffered Saline Solution (without phenol red or sodium bicarbonate)
Store at 4°C covered with foil

Hanks Buffered Saline Solution (without phenol red and sodium bicarbonate)

5.4mM KCl
0.3mM Na_2HPO_4
0.4mM KH_2PO_4
1.3mM $CaCl_2$
0.5mM $MgCl_2$
0.6mM $MgSO_4$
137mM NaCl
5.6mM D-glucose
pH 7.4
Make up to 1L with dH_2O
Store at 4°C

Mycoplasma fixative

1 part glacial acetic acid and 3 parts methanol

Mycoplasma mounting fluid

22.2ml 0.1M citric acid
27.8ml 0.2M $Na_2HPO_4 \cdot 2H_2O$
50ml glycerol
pH 5.5
Store at 4°C

B2: MTT assay

MTT reagent 5mg/ml

Weigh out 100mg MTT
Add 20ml sterile PBS in TC hood
Vortex the mixture and incubate in the water bath at 37°C for 15 minutes
Filter the solution into another 50ml tube through a 0.2µm filter
Wrap the solution in foil and store at 4°C

Solubilisation reagent

Dissolve 50g of SLS in 500ml of dH2O
Add 153.2µl of conc. HCl
Store at room temperature

B3: Cell Cycle

RNAse 10mg/ml

Dissolve pancreatic RNAse (RNAse A) in 10mM Tris.Cl pH7.5 and 15mM NaCl
Heat at 100°C for 15 minutes and cool slowly to room temperature
Make aliquots and store at -20°C

PI (Propidium iodide) staining solution (make up fresh always)

0.1% Triton X100
2mM MgCl2
100mM NaCl
10mM PIPES buffer
10µg/ml Propidium iodide
Make up to 50ml with dH_2O

B4: Western blot

RIPA buffer

150mM NaCl
1% Triton X100
0.1% SDS
10mM Tris pH 7.5
1% Na deoxycholate
Make up to 200ml with dH_2O
Store at 4°C
Add Protease inhibitor and phosphatase inhibitor fresh every time before use

5x loading buffer

15.1g Tris
72g glycine
Dissolve in 50ml dH_2O
pH to 6.8
add 5g SDS
Make up to 100ml with dH_2O
Store at room temperature and heat before use

5x Laemmli dye

200µl 5x loading dye
100µl β-mercaptoethanol
100µl 0.025% Bromophenol blue
Store at -20°C

1M Tris pH 8.8

60.5g Tris
Add 300ml dH_2O
pH to 8.8
Make up to 500ml with dH_2O
Autoclave
Store at room temperature

10% Resolving gel

5.5ml dH2O
7.5ml 1M Tris pH 8.8
200µl 10% SDS
6.7ml 30% Acrylamide (Sigma)
400µl 10% APS
40µl TEMED

15% Resolving gel

2.2ml dH2O
7.5ml 1M Tris pH 8.8
200µl 10% SDS
10ml 30% Acrylamide (Sigma)
400µl 10% APS
40µl TEMED

4% Stacking gel

7.3ml dH2O
1.25ml 0.94M Tris pH 6.7
100µl 10% SDS
1.3ml 30% Acrylamide (Sigma)
120µl 10% APS
12µl TEMED

10x Running buffer

29g Tris
144g Glycine
10g SDS
Make up to 1L with dH_2O
Store at room temperature

10x Transfer buffer

144g Glycine
38g Tris
Make up to 1L with dH_2O
Store at room temperature

10x Tris Buffered Saline (TBS)

60.5g Tris
87.6g NaCl
pH 7.5
Make up to 1L with dH_2O
Store at room temperature

Stripping buffer

1.4ml beta-mercaptoethanol
40ml 10% SDS
12.5ml 1M Tris pH 6.7
Make up to 200ml with dH_2O
Store at 4°C

B5: Thioredoxin reductase

Mitochondrial isolation buffer

20mM HEPES-KOH
10mM KCl
1.5mM $MgCl_2$
1mM EDTA
1mM EGTA
0.25M Sucrose
Add 100ml of dH_2O
pH 7.5
Make up to 200ml with dH_2O
Filter sterilise and store at 4°C

NOTE: CHAPS and protease inhibitor should be added fresh on the day of the experiment

Lysis buffer

40mM HEPES-KOH
50mM NaCl
1mM EDTA
1mM EGTA
Add 100ml dH_2O
pH to 7.4
Make up to 200ml with dH_2O
Filter sterilise
Store at 4°C

NOTE: CHAPS and protease inhibitor should be added fresh on the day of the experiment

PE buffer

100mM Potassium phosphate
10mM EDTA
pH 7
Make up to 200ml with dH_2O
Autoclave / Filter sterilise
Store at 4°C

B6: ROS

DCFDA

Make up fresh from primary stock
Molecular weight = 487.26g/mol
Primary stock = 50mM in DMSO (50mg in 2.05ml)

Kreb's ringer (KR) buffer

Table 5 KR buffer solution table: A stock solution without glucose must be made up, pH to 7.4, filter sterilised and stored at 4°C. Glucose must be added fresh on the day of the experiment.

Reagent	Molar mass	Final conc.	Mass for 1L	Vol. For 500ml	Vol. For 200ml
NaCl (5M)	57.75	110mM	6.35g	11ml	4.4ml
KCl (1M)	74.56	2.6mM	0.19g	1.3ml	0.52ml
$MgSO_4$ (1M)	120.36	1.2mM	0.14g	0.6ml	0.24ml
KH_2PO_4 (1M)	136.09	1.2mM	0.16g	0.6ml	0.24ml
$NaHCO_3$ (1M)	84.01	25mM	2.10g	12.5ml	5ml
Glucose	180.08	11mM	1.98g	0.99g	0.396g

Crystal Violet

1% crystal violet
50% Methanol
Store at room temperature